Production and preservation of boar spermatozoa.

Thesis

PRODUCTION AND PRESERVATION OF BOAR SPERMATOZOA

by

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A dissertation submitted to the Open University
for the degree of
Bachelor of Philosophy

Faculty of Science
Open University

April 1979

Date of submission: 19.4.79
Date of award: 5.2.80

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PREFACE

These studies are original work by the author and any assistance from others is specifically acknowledged. No part of the dissertation has been submitted to any other University for any degree or diploma.

Charles William Wood

April, 1979

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor, Dr. Peter Hogarth for his constant advice and encouragement during the preparation of this dissertation.

I am greatly indebted to the Winston Churchill Memorial Trust for the award of a Churchill Travelling Fellowship, to visit North America, to study the work being undertaken on the artificial insemination of pigs in Canada and the United States.

I wish to acknowledge my gratitude to the Meat and Livestock Commission for the facilities to complete the experimental work, for access to the literature used in the preparation of this dissertation, and especially to Dr. H.C.B.Reed for his kind support.

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ABSTRACT

The use of artificial insemination for breeding pigs has developed slowly over the past few years within the United Kingdom. Future development will depend to a large extent, on the ability to maintain and improve on the present conception rates, by improved techniques for the preservation *in vitro* of boar spermatozoa.

This dissertation examines the production, and the methods of preservation of boar spermatozoa. In Section I, the production of spermatozoa is reviewed, from the division of the germ cells within the seminiferous epithelium, through the various stages of development, to the maturation of the spermatozoa within the epididymis.

Variation in sperm production rates and sperm output are related to age and strain of boar, ejaculation frequency and environmental temperature. These aspects are examined, as are the methods of collection of semen from the boars and the laboratory examination of the spermatozoa.

In Section II, a review is undertaken of the methods of extending the life of the spermatozoa when stored *in vitro*. The advantages of storing spermatozoa 'fresh' as opposed to 'frozen' are discussed, and the various diluents used for storing spermatozoa by each method are examined.

The freezing of boar spermatozoa has presented more difficulties than the freezing of bull spermatozoa. The various procedures involved with freezing, including the effects of glycerol, 'holding time' and the use of surfactant agents are investigated.

Two experiments are reported. The 1st. experiment investigates the relationship of morphological abnormalities of spermatozoa to conception rates and litter size. The 2nd. experiment examines the effect on acrosomal integrity of the various stages involved with cooling and freezing the spermatozoa.

THE PRODUCTION AND PRESERVATION OF BOAR SPERMATOZOA

Introduction

A boar kept under commercial farm conditions should be capable of serving 25 sows twice per year, producing some 500 piglets. This is assuming that two litters are produced per year with an average litter size of ten. In practice, this is difficult to achieve. Modern methods of organized production usually depend on serving sows in batches which necessitates using the boar exhaust -ively for a short period followed by a period of idleness, during which time he still has to be housed and fed. Other factors affect his output such as periods of infertility, refusal to work, lameness, susceptibility to disease and the inability when fully grown to serve small sows that are unable to take his weight. Some of these factors can be overcome by careful management, regular veterinary inspection and the use of a service crate to take the weight off the sow. The problem still remains that purchasing and maintaining a boar is expensive and involves considerable risk.

In Great Britain, the artificial insemination (A.I.) of pigs has slowly become established over the past 25 years. In 1955, three cattle insemination centres offered a pig A.I. service with disappointing results; only 18 % of 951 sows inseminated farrowed to a first insemination. By 1970, the number of inseminations had risen to 62,000 and the conception rate to 70 %. With the outbreak of foot and mouth disease in 1973, which involved restrictions being placed on the movement of animals, the number of inseminations carried out, escalated to over 90,000.

Over the past few years in Great Britain, the number of pigs artificially inseminated as a percentage of the total number of

inseminations, varies between 4 to 6 %. A typical year is 1974, when approximately 5 % of inseminations were by A.I.

No. of breeding pigs (Great Britain-1974)	-	828,000
No. of inseminations (1.8 per pig per year)	-	1,490,400
No. of pigs artificially inseminated	-	72,567

Willems (1978) investigated by means of a questionnaire sent to 26 countries, the development of the artificial insemination of pigs in Western European countries. He concluded that during the past few years, the use of A.I. in pigs has been expanding rapidly, although in most countries it is still of only minor importance in total pig production.

There are several factors that influence the use of artificial insemination of pigs by the farmer. Probably the most important is the costs incurred in using A.I., which should be comparable to and preferably less than the costs of natural mating. Semen should be available for A.I. from boars with major heritable genetic traits that are of economic importance and an efficient service should be available to supply high quality tested semen from a selection of boars of the major breeds. It is also important that the insemination technique and apparatus for 'on the farm' use, should be simple and reliable, so that inseminations can be undertaken by a competent stockman.

Advantages of the use of A.I.

Livestock improvement Boars are selected by progeny testing stations for superior physical conformation and food conversion rates. Having established the boars'superiority, the role of A.I. is the wide dissemination of the genetic material of the boar. It is estimated that by A.I. one boar can be used on 2,000 sows with a potential of 20,000 progeny (Melrose 1966). The close relationship between progeny testing station and artificial insemination centre is essential both for the availability of 'superior' boars, also for progeny

testing these boars to assess the heritability of their characteristic traits.

Disease control The most frequent and the most efficient method of spread for all infectious diseases is by pig to pig contact. The introduction of new blood lines into a herd can be overcome by the use of artificial insemination, the risk of introducing infection with semen being considerably less than that associated with the introduction of a boar to a farm. The prevention of the spread of brucellosis (Vergote de Lantsherre, Van Snick and Lejeune 1964; Brone 1966) and foot and mouth disease (Rutgers 1966) by the use of A.I. has been reported. Outbreaks of foot and mouth disease or Swine Vesicular disease are accompanied by widespread movement of animal restrictions, the difficulties resulting from these restrictions can be alleviated by the use of A.I.

Boars used for artificial insemination are subjected to strict health tests and kept in isolation from other stock.

Measure of genetic improvement The long term storage of boar semen can be used as an index to measure the rate of genetic improvement. When semen is stored for several years and eventually used, the resulting offspring can be compared to offspring from a future generation boar.

Export The export of semen is simpler and cheaper than transporting live animals.

The ability to preserve boar semen in vitro is a necessary pre-requisite of the development of artificial insemination services. The constant need for improved standards, provides a powerful stimulus for the morphological as well as the chemical investigation of boar semen. Over the past few years, particularly since the work at Cambridge by Polge and Rowson in the mid 1950's, there has been a

rapidly increasing number of publications in this field.

The object of this dissertation is to look at the subject of boar spermatozoa, their development from the germ cells in the seminiferous epithelium, through the maturation processes in the epididymis, and the methods of storage in vitro. The techniques used to collect semen from the boar are examined, also the methods used to assess semen 'quality' in the laboratory before dilution and storage.

The ultimate objective of the storage of semen is to retain the viability of the cells so that when they are used for artificial insemination they will achieve high conception rates and large litters of piglets. A basic knowledge of the production and preservation of boar semen is a requirement for the development of preservation methods, which are essential for the continued growth of the use of artificial insemination within the pig industry.

SECTION I - THE PRODUCTION OF BOAR SPERMATOZOA

SPERMATOGENESIS AND THE DEVELOPMENT OF THE SPERMATOZOA

Introduction

The viability of spermatozoa produced at ejaculation must depend on the processes of development which occur within the seminiferous epithelium, from the initial production of the spermatogonia from stem cells, through the various stages of spermatogenesis to the release of the spermatozoa, and the final attainment of maturity of the spermatozoa within the cauda epididymis.

The development of the spermatozoa within the seminiferous tubules has been studied over the past 100 years, the principal workers being Brown(1885), Von Ebner (1888), Regaud (1901), Retzius (1909), Curtis (1918), Stieve (1930) and many others. It was not until the early 1950's that a detailed classification of the various stages of sperm cell development was undertaken, primarily on laboratory animals by Roosen-Runge and Giesel (1950) and Leblond and Clermont (1952). This was followed by studies on the various stages of spermatogenesis in domestic animals, particularly of bovine spermatozoa which were becoming used extensively for artificial insemination (Knudson 1954,1958; Kramer 1960; Hochereau 1963) and for the boar (Henricson and Blackstrom 1963; Kennelly 1960; Kennelly and Foote 1961, 1964; Swierstra 1968).

Cellular Elements of the spermatogenic cycle

Spermatogonia are the germ cells which originate from the gonocytes and are contained in the parietal layer of the seminiferous tubules. Ortavant et al (1969) defines them as 'the combination of germ cells contained in the parietal layer of the seminiferous epithelium, the last generation of which gives rise to primary spermatocytes'.. Primitive type A spermatogonia appear shortly after

birth and are referred to as stem cells. When the animal reaches sexual maturity, the stem cell undergoes repeated mitotic divisions, from which are derived the A_2, Intermediate and B type spermatogonia. (Fig 1). New stem cells arise to replace those which develop in the spermatogenic series, the origin of which is probably the first or second mitotic division (Ortavant et al. 1969). Because of the characteristic appearance of the nucleus of the A type cells they are usually described as 'dust' like spermatogonia as opposed to the 'crust' like appearance of the type B spermatogonia (Regaud 1901).

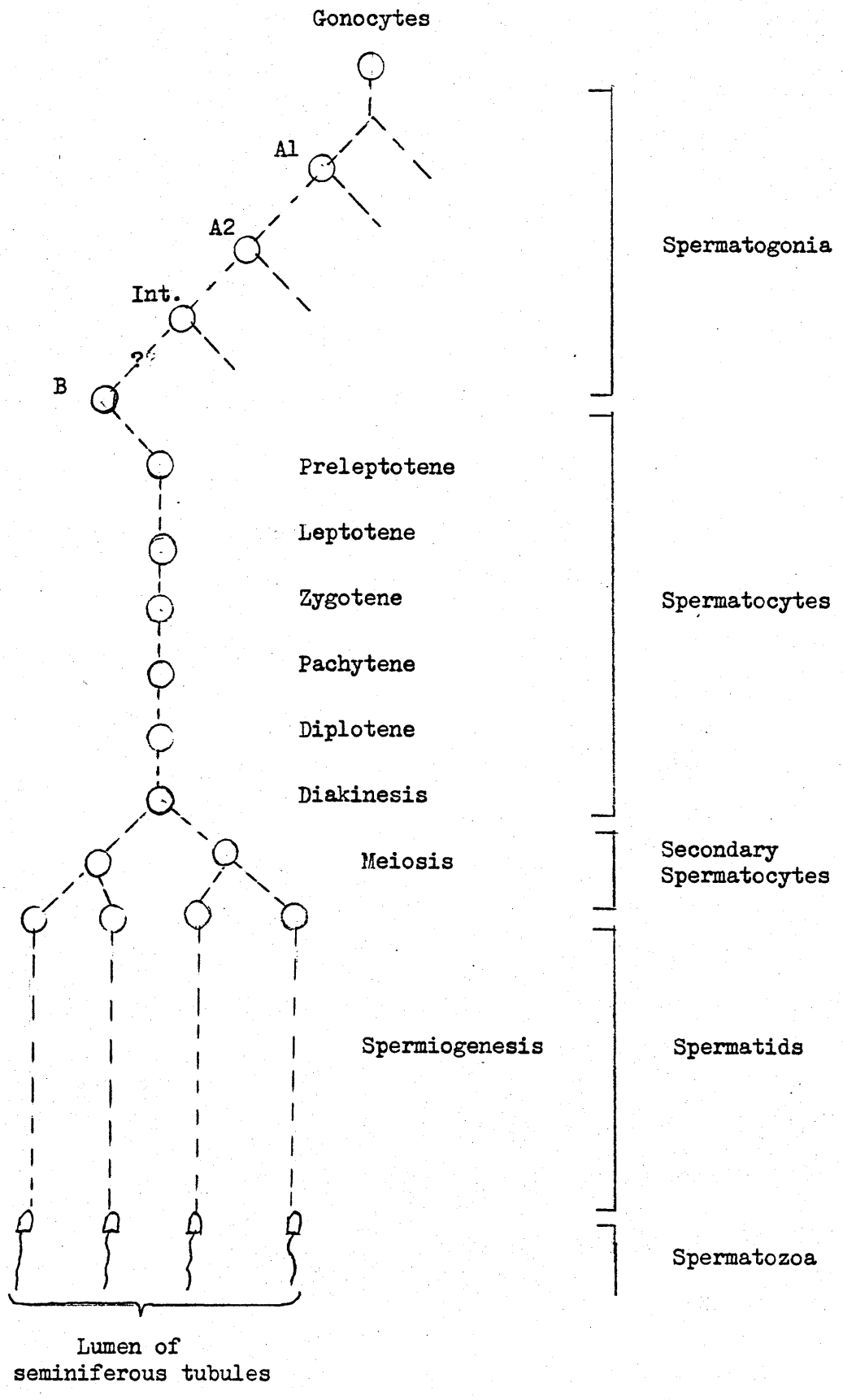
For boars, the beginning of spermatogenesis cannot clearly be defined, since the scheme for spermatogonial divisions has not yet been determined (Swierstra 1968b). Although the A, Intermediate and B types are clearly distinguishable (Henricson & Backstrom 1963).

The spermatogonia proliferate on the same site of the seminiferous tubule, so that a cross-section of the tubule shows several superimposed generations of germ cells at the basement membrane. As meiosis proceeds the primary spermatocytes move towards the central part of the seminiferous tubule.

The meiotic division is preceded by the preleptotene stage during which active synthesis of DNA occurs; the cell then continues the classic stages of meiotic prophase through the leptotene, zygotene, pachytene, diplotene and diakinesis to completion before entering the rapid metaphase, anaphase and telophase to complete the 1st. meiotic division, which gives rise to two secondary spermatocytes.

The secondary spermatocytes contain a haploid set of chromosomes. During the meiotic division, separation of the sex chromosomes occurs, resulting in two categories of secondary sperm--atocytes; those bearing an X chromosome and those containing a Y chromosome. After a short interphase the secondary spermatocyte divides to give rise to two spermatids.

Diagram to illustrate the Spermatogenic Sequence in the boar



(Fig 1)

The metamorphosis of the spermatids

The spermatids derived from the secondary spermatocytes undergo a complex morphological transformation and modification of their components. This process is called spermiogenesis or spermateleosis (Bishop and Walton 1960). The several stages of the development of the acrosome are clearly distinguishable with a light microscope using the periodic-acid-Schiff (PAS) reaction; this stains the glycoprotein material deeply red in the acrosomal structure. The stages of development of the acrosome can be used as a basis for the precise classification of the seminiferous epithelium cycle (Leblond and Clermont 1952).

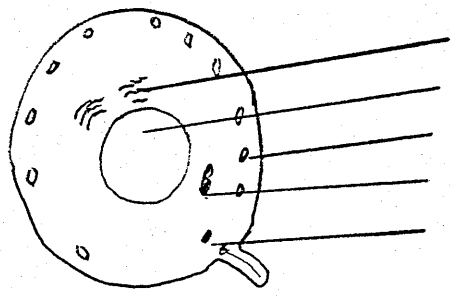
The nuclear and cytoplasmic changes that occur during spermiogenesis govern to a great extent the quality of the final product (Ortavant et al.1969). The sequence of transformations is subdivided into four phases. These are the Golgi phase, cap phase, acrosomal phase and the maturation phase. (Fig.2).

Golgi phase Proacrosome granules in the Golgi region of the cell gather together to form a single acrosomic granule contained within an acrosomic vesicle. The structure is located close to the nucleus of the young spermatid at the future anterior pole. At the same time, one of the two centrioles, located at the opposite pole, gives rise to the flagellum. This will grow out of the cell though still remaining enclosed within the plasma membrane (Fig 2).

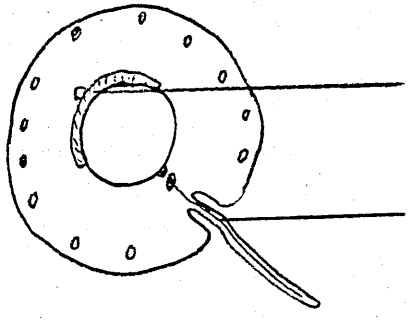
Cap phase The acrosomic granule develops and flattens on the nucleus of the spermatid. The Golgi apparatus separates from the acrosomic vesicle and migrates to the caudal part of the cell. The two centrioles migrate towards the posterior pole of the nucleus. The proximal centriole becomes localised at this position of the future junction of the head and tail and could possibly represent the origins of the flagellar beat (Monesi 1972). The distal centriole acts as

Diagrams to illustrate the Metamorphosis of a Spermatid

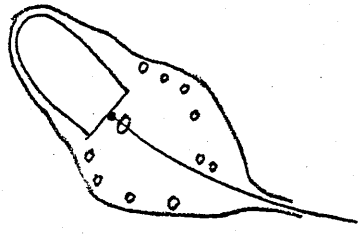
Golgi phase



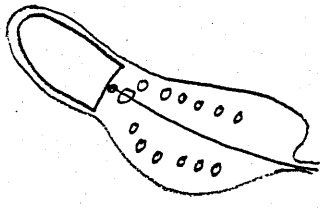
Cap phase



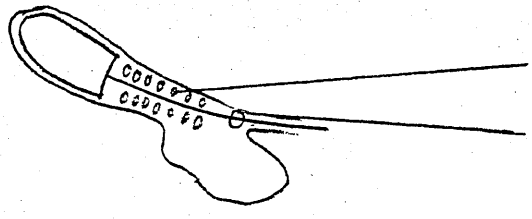
Acrosomal phase



Nucleus moves to periphery of cell



Cytoplasm is displaced



(Fig 2)

a basal corpuscle for the flagellum.

Acrosome phase The acrosomal vesicle covers the nucleus increasingly as the nucleus undergoes elongation. The granule now occupies all the acrosomic vesicle. The nucleus of the spermatid moves from the centre to the periphery of the cell and comes into contact with the cell membrane. The cytoplasm is displaced behind the caudal pole of the nucleus and elongates to surround the proximal part of the flagellum (Monesi 1972).

The mitochondria in the cytoplasm migrate to surround the flagellum; these will develop later into the mitochondrial sheath. The chromatid body moves to the distal centriole to form the annulus ring around the flagellum. This will later migrate to the junction of the mid-piece and tail at the distal end of the mitochondrial sheath.

The developing spermatid within the seminiferous epithelium, rotates during the acrosome stage so that the acrosome and nucleus become orientated towards the nuclei of the Sertoli cells and the tail towards the lumen.

Maturation phase During the maturation phase the spermatid completes its transformation into a spermatozoon. The developing acrosome finally covers two thirds of the anterior of the nucleus of the spermatozoon. The nucleus is now surrounded successively by a nuclear membrane, internal acrosome membrane, acrosome body, external acrosome membrane and the cytoplasmic membrane (Courot, Hochereau de Reviers and Ortavant 1970). The ability to retain the integrity of these membranes is particularly important in the preservation of boar spermatozoa in vitro.

During the maturation phase, nine coarse 'outer fibres' are formed around the axial filaments. These fuse to form a sheath around the centrioles and to form the wall of the connecting piece

to the neck. In the mid-piece, these fibres are surrounded by the mitochondria which arrange themselves helically forming the mitochondrial sheath. At the distal part of the flagellum, a fibrous sheath is formed around the outer fibres.

Towards the end of the maturation phase, the axial filament that originates from the proximal centriole, constitutes 2 tubular fibrils that are surrounded by 9 doublets, an arrangement that is a common feature of flagellated cells.

Sertoli cells

Sertoli cells are the somatic elements of the seminiferous epithelium. They are large cells that undergo changes in shape, size and activity in relation to the seminiferous epithelium cycle. The basal part of these cells rest on the seminiferous tubule wall and the apex extends inwards towards the lumen. By their particular position, their structure and their intense metabolic activity, the Sertoli cells play a significant role in the structure of the seminif -erous epithelium, the metabolic exchanges with the germ cells, and the coordination of spermatogenesis (Courot et al. 1970).

Extrusion of spermatozoa into the lumen

As the spermatozoa develop they orientate their acrosomes towards the nuclei of the Sertoli cells. The anterior portions of the spermatid head becomes lodged in shallow crypts in the surface of the Sertoli cell. Slender processes from the Sertoli cell extend outward and appear to grasp the residual cytoplasmic bodies of the spermatid. (Fawcett and Phillips 1969). This grasp is maintained as the spermatid appears to be actively extruded into the lumen of the seminiferous tubule. When the stalk of cytoplasm connecting the spermatozoon to the Sertoli cell is finally broken, part is engulfed by the Sertoli cell whilst the other part rounds up to form the cytoplasmic droplet on the neck region of the spermatozoon

The seminiferous epithelium cycle

In any given segment of the seminiferous tubule, the stem cells initiate at regular intervals, the series of mitotic divisions preceding meiosis. Once this process has been initiated, each step of spermatogenesis has a fixed constant duration, so that in a given area of the seminiferous epithelium can be observed a precise number of distinct cellular associations; each consisting of one or two generation of spermatogonia, spermatocytes and spermatids. The combination of the different generations, changes with time as spermatogenesis proceeds. The sequence of events occuring from the disappearance of a given cellular association to its reappearance constitutes one cycle of the seminiferous epithelium (Leblond and Clermont 1952).

In the development from stem cell to spermatozoa, the number of cycles varies according to species and the definition of the starting point of spermatogenesis. When the first of a series of spermatogonial divisions is taken as the starting point, then spermatogenesis extends over 4 cycles in the rat (Leblond and Clermont 1952), the mouse (Oakberg 1956), the ram and bull (Ortavant 1959) the boar (Swierstra 1968b) and 6 cycles in the monkey (Clermont and Leblond 1959).

Swierstra (1968b) measured the duration of spermatogenesis in the boar using tritiated thymidine which emits low energy particles with high resolution autoradiography (Crathorn and Shooter 1960; Rubini, Cronkite, Bond and Fliedner 1960; Monesi 1962). Swierstra estimated the duration of spermatogenesis in the boar to be 34.4 days; as this extends over four cycles, the duration of each cycle was 8.6 $\pm$ 0.1 days.

The arrangement of the germ cells in fixed combinations of cellular generations enables the cycle of the seminiferous epithelium to be divided into identifiable component stages.

The cycle of the boar seminiferous epithelium can be divided into eight distinct stages. Each stage can be readily identified under the low power of a light microscope by a trained technician. The breakdown into the several stages of the cycle facilitates rapid identification of the principal events and is an important pre-requisite for most quantitive investigations of spermatogenesis (Berndtson 1977). The stages are based on the meiotic divisions, variations in shape of the spermatid nucleus and the release of spermatozoa into the lumen of the seminiferous tubule (Curtis 1918; Roosen-Runge and Giesel,1950; Ortavant 1959; Swierstra and Foote 1963; Swierstra 1968b; Ortavant et al 1969).

The stages can be identified as follows:-

Stage 1 From the absence of spermatozoa in the lumen of the tubule to the onset of elongation of the spermatid nuclei.

Stage 2 From the beginning of elongation to the end of elongation of the spermatid nuclei.

Stage 3 From the end of elongation of the spermatid nuclei to the beginning of the 1st meiotic division.

Stage 4 From the beginning of the 1st meiotic division to the end of the 2nd meiotic division.

Stage 5 From the end of the 2nd meiotic division to when the chromatin in the new spermatids shows a 'dusty' appearance.

Stage 6 From when the spermatid nuclei show a 'dusty' appearance to the time when all the spermatozoa have left the Sertoli cells and move towards the lumen of the tubule.

Stage 7 From the beginning to the end of the migration of the spermatozoa towards the lumen.

Stage 8 From when spermatozoa line the lumen to their complete disappearance from the lumen.

Relative Fequencies and Durations of the Eight Stages of the Cycle of the Seminiferous Epithelium of the Boar Table 1

	Sources		
	Ortavant(1959)	Swierstra(1968b)	Ortavant et al(1969)
Stage			
1	10.7	10.8 ± 0.3	12.7 ± 1.3
2	16.9	14.4 ± 0.3	16.7 ± 1.4
3	2.1	3.5 ± 0.2	5.0 ± 0.5
4	13.7	11.6 ± 0.3	13.8 ± 1.0
5	10.7	8.9 ± 0.3	9.7 ± 1.2
6	20.9	20.3 ± 0.4	19.0 ± 1.0
7	11.6	18.5 ± 0.4	12.6 ± 1.6
8	13.5	12.0 ± 0.3	9.5 ± 2.0
	100.1	100.0	100.0

The relative frequency of each stage of the seminiferous epithelial cycle varies between species; within a species the frequencies are relatively constant among animals (Hochereau 1963a; Swierstra 1968b; Ortavant et al.1969). In Table 1, the eight stages of the cycle in the seminiferous epithelium of the boar, from three different sources, are basically in agreement. The differences could be due to using material from different strains of animals, or by the different treatment of the testes material. Elongated spermatids are often highly susceptible to treatment effects (Eschenbrenner, Miller and Lorenz 1948; Clermont and Morgentaler 1955; Clermont and Harvey 1967; Berndtson and Desjardins 1974). The most noticeable difference in Table 1 is at stage 7. This stage is identified by the migration of

the spermatozoa towards the lumen. The discrepancy could possibly be accounted for by a different interpretation by the investigators of this stage, as the stages of the cycle are arbitrary, man-made divisions of a continuous process, rather than a biological constant for the species.

Spermatogenic wave

Successive stages of spermatogenesis are arranged next to one another along the length of the seminiferous tubule; a complete series of these stages then forms the spermatogenic wave. The coordinating factor involvedin the synchronization and imposing order on this succession of waves is unclear. It could be the testicular fluid that flows towards the rete testis (Perey, Clermont and Leblond, 1961) or possibly the Sertoli cells facilitating the passage of signals through the areas of the germinal epithelium (Roosen-Runge 1962).

The spermatogenic cycle of the boar. Four cycles of the seminiferous epithelium are shown, and the duration of the eight stages comprising each cycle are indicated. Source - Swierstra (1968 b)

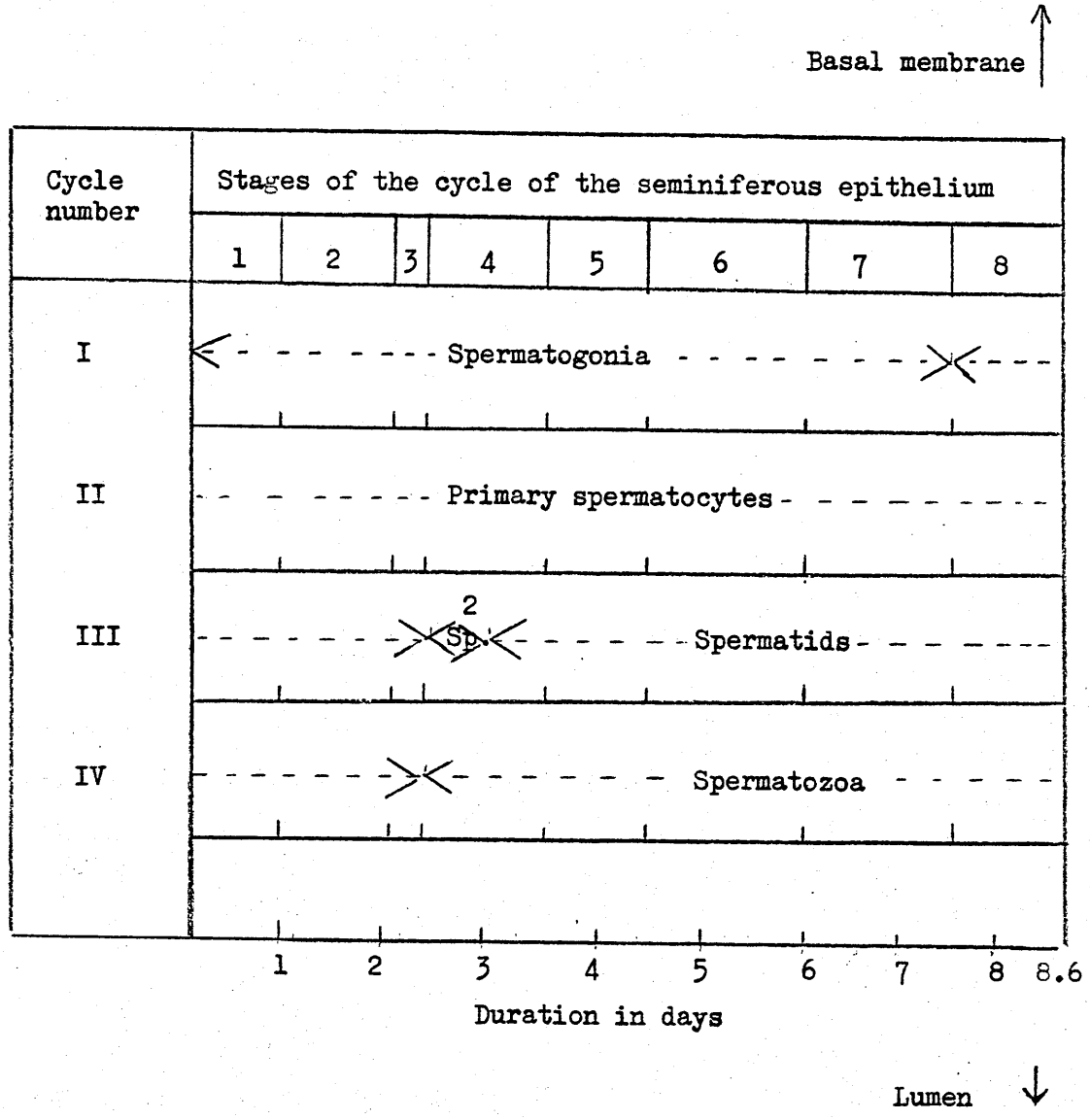


Fig 3. Diagrammatic representation of the dynamics of spermatogenesis in the boar. Starting from the top left and proceeding to the right, one can complete the entire development from stem cell to spermatozoon. over the four lines. Each line corresponding to one cycle of the seminiferous epithelium. The lines are subdivided to represent the stages of the cycle.

(Fig. 3)

THE FUNCTION OF THE EPIDIDYMIS

Introduction

The composition of semen and the epididymal contents has been studied extensively in various species including rat, rabbit, bull and ram. The composition of boar semen and the epididymal contents has been the subject of comparatively few investigations. The most notable of these are McKenzie et al (1938); Mann (1964); Crabo (1965); Einarsson (1971) and Swierstra (1971).

McKenzie et al (1938) studied the relative contributions of the accessory glands to the semen by systematically excising the glands which were accessible to such an operation. The drawback to this approach is the influence of the operation on the subsequent production and performance of the remaining organs.

In his classic work 'The Biochemistry of Semen and the Male Reproductive Tract', Mann (1964) gives an account of the structure of the reproductive organs and a biochemical analysis of boar semen.

Crabo (1965) investigated the contents of the boar epididymis using material from selected boars that were slaughtered and castrated. He outlined a technique suitable for the analysis of plasma and sperm from different regions of the epididymis. By analytical studies of the plasma from different segments of the duct he was able to investigate the function of the epididymal epithelium in different segments of the organ.

Einarsson (1971) collected information on the composition of the cauda epididymis content. He studied the relative contribut -ions to the ejaculate of the accessory sex glands and the composition of the seminal plasma under different hormonal influences.

Swierstra (1971) investigated the sperm production of boars by measuring the epididymal sperm reserves after depletion of the reserves.

The epididymis is situated close to the testis, on the dorsal side. Its basic function is to convey the spermatozoa from the testis to the ejaculatory duct. During the passage through the epididymis, the spermatozoa undergo changes both in structure and function which collectively are said to represent a process of maturation. A further function of the epididymis is that it acts as a sperm reservoir (Martan 1969). During periods of sexual rest spermatozoa accumulate at the distal end of the duct where they can be stored for a long time without loss of fertilizing power (Bishop and Walton 1960).

Conveyance of sperm from the testis to the ejaculatory duct

The spermatozoa, on leaving the seminiferous tubules, pass along a series of collecting ducts that comprise the rete testis. The rete testis communicates with some 14-21 convaluated efferent ductules at the most proximal part of the epididymis. These ductules converge to form a single convaluted duct which in the boar extends to some 62 - 64 meters (Crabo 1965). The convolutions of the duct are enclosed within a sheet of connective tissue forming a short, cord-like structure which constitutes the epididymis. The epididymis may be divided topographically into three distinct regions; the caput at the proximal end, the corpus which comprises the middle section, and the cauda epididymis at the distal end. (Fig 4).

The spermatozoa are transported from the testis in a fluid. Some of the fluid is secreted from the seminiferous tubules but as the tubules are two-ended and both ends open into the rete testis, then rete fluid may enter at one end and be expelled from the other. A valve-like mechanism at the ends of the tubuli recti may prevent the back flow of the spermatozoa and only allow the entry of the fluid (Roosen-Runge 1961). The continuous flow of the fluid may be aided by the cilia of the efferent ductules (Crabo 1965), or by the

Diagram of cross section through testis and epididymis

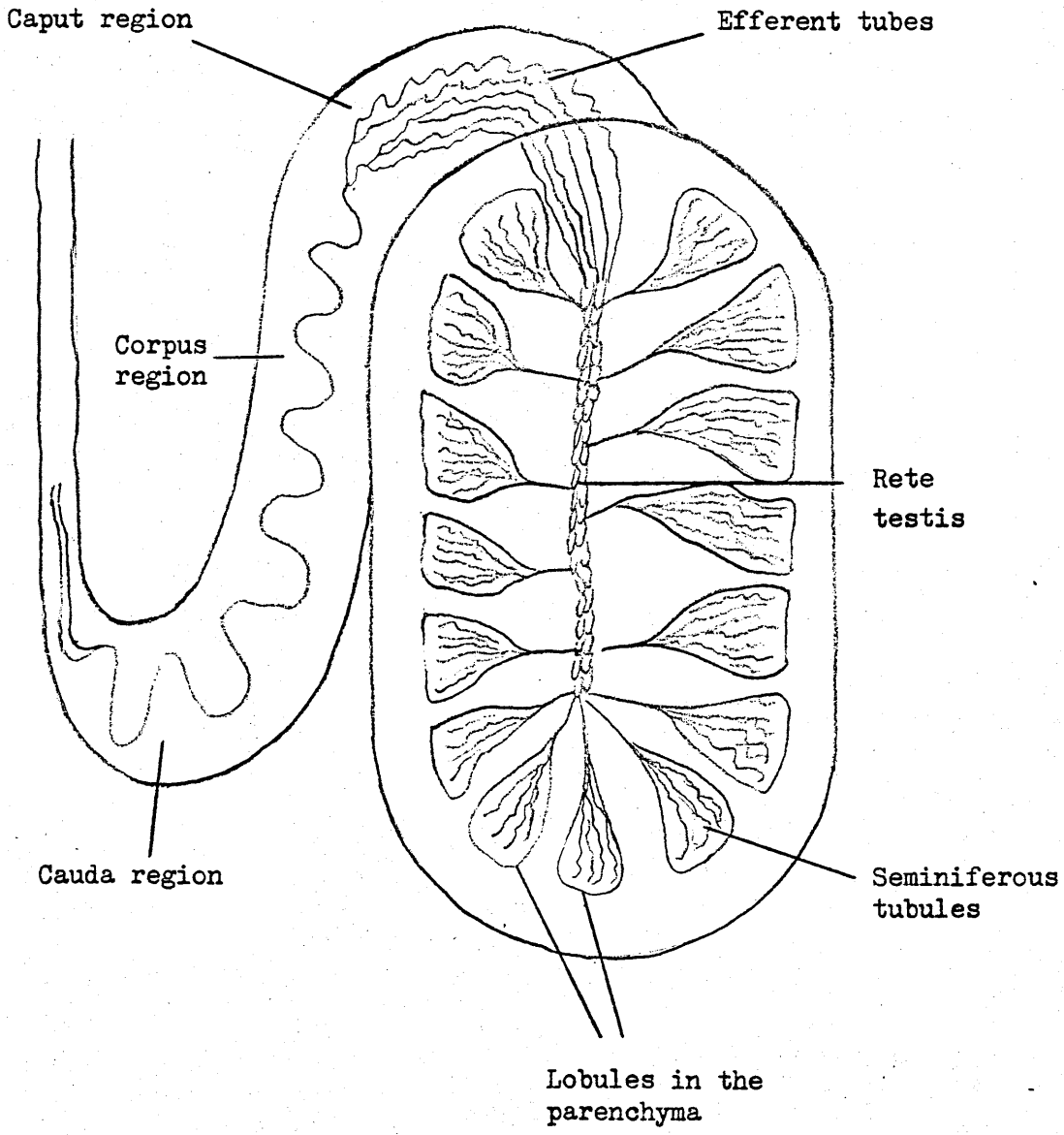


Fig. 4

peristaltic action of the contractile cells in the peritubular tissue (Setchell and Waites 1971). Crabo (1965) estimated that 99.7 % of the rete fluid that leaves the testis is reabsorbed in the head region of the epididymis. Setchell et al (1971) postulated that if a quantity of some substance was discharged into the fluid with the release of each spermatozoon, then the reabsorption of the fluid in the head of the epididymis would provide a logical method for information on the rate of spermatogenesis to feedback to the pituitary.

The spermatozoa on entering the caput region of the epididymis are neither structurally nor functionally complete; samples extracted from this region are not motile (Schersten 1937, from Crabo 1965). The transport of the spermatozoa through the epididymis is facilitated by the peristaltic, pendular and segmentation movements of this organ (Risley and Turbyfill 1957).

Using H^3 thymidine to label spermatozoa, Swierstra (1968b) calculated the time taken for the transit of the spermatozoa through the epididymis to range from 9 to 12 days (mean 10.2 days). He compared this with the estimated time of 14 days reported by Singh (1962). The difference he attributed to the lower frequency of collections from the boar made by Singh. A more intensive semen collection schedule would result in the earlier detection of labelled spermatozoa in the ejaculate.

Sperm maturation

The changes to the structure and function of the spermatozoa during their passage through the epididymis result in the spermatozoa aquiring the ability to fertilize and a capacity for vigorous motility. The most obvious changes to the sperm morphology are the migration of the cytoplasmic droplet, the marked reduction in acrosome size and changes in the nuclear material.

Cytoplasmic droplets Hancock (1957) investigated the incidence of

spermatozoa with cytoplasmic droplets at different levels of the epididymis. The material he used was obtained from two boars, killed at a slaughterhouse, and the epididymis attached to the testis removed and fixed. Hancock observed that in the caput region, all the spermatozoa had cytoplasmic droplets, either neck droplets or distal droplets. The spermatozoa with neck droplets were prominent at the proximal region of the caput epididymis and the spermatozoa with the distal droplets and those without droplets were prominent in the cauda region and vas deferens.

The cytoplasmic droplet appears to migrate from the neck region to the distal part of the mid-piece of the tail of the spermatozoa as it passes through the epididymis, before its subsequent removal from the tail piece. Should an animal be collected from too frequently then the incidence of spermatozoa in the ejaculate displaying the cytodroplet will rise, indicating the presence of immature sperm cells (Monesi 1972).

Observation of the position of the droplets on the mid-piece of the spermatozoa show that they are almost always found in either one of two regions, i.e. at the neck or at the distal region. The 'bent tail' abnormality (fig.10) displays a droplet at the bend of the tail indicating a possible relationship between the bent tail and the distal droplet (Lasley & Bogart 1944). The bent tail abnormality could possibly be the result of the bending action of the spermatozoa during the translocation of the distal droplet (Saake 1970).

Investigation of the structure of the boar cytoplasmic droplet was undertaken by Bloom & Nicander (1961) using electron microscopy. They reported that the dense cytoplasm contained by the droplet consists of many tubular or lamellar elements similar to those observed in the cytoplasmic droplets from other species. The source of these internal structures is probably the disintegrating

Golgi apparatus and the endoplasmic reticulum of spermatids. Bloom & Nicander (1961) observed that there was no structural modification of the mid-piece of the spermatozoa in connection with the movement of the droplet. The droplet is bounded by the cell membrane and its contents borders on the neck and mid-piece.

The function of the droplet is unknown. Some authors regard it as simply a remnant of the spermatid cytoplasm and of no special interest (Mann 1964). Harrison & White (1972) demonstrated that significant levels of some glycolytic enzymes are present in cytoplasmic droplets as well as in spermatozoa. The levels of glycolytic enzymes found in the seminal plasma corresponds to the number of droplets that have disappeared. It is possible that most, if not all of the glycolytic enzyme activity in seminal plasma could be traced to disintegrating cytoplasmic droplets.

White & Wales (1961) reported that the most notable difference between the spermatozoa from the ejaculate and the spermatozoa from the epididymis was the resistance of the epididymal spermatozoa to 'cold shock'. This was particularly true of spermatozoa with attached droplets. If the presence of an attached droplet can be regarded as evidence of immaturity, then this immaturity rather than the droplet per se, may be the important factor in determining the susceptibility of spermatozoa to cold shock.

Reduction in acrosome size The appearance of the spermatozoa using a light microscope shows virtually no change to their outline from when they leave the testis and during their passage through the epididymis. This is because the spermatozoal heads of most mammals are flattened; they tend in smears, always to present their broad surface to the microscopist. Using electron microscopy, the spermatozoa are first embedded then sectioned, so that sagittal sections of sperm heads show, in profile, features of the membranes

that are difficult to observe in smears (Fawcett & Phillips 1969).

Jones (1971) examinined the structural changes to the heads of boar spermatozoa during their passage through the epididymis using an electron microscope. He observed an overall reduction in the size of the acrosome as the adjacent acrosome and nuclear membranes moved into closer apposition and the material forming the perforatorium condensed. Using this criterion to score whether a spermatozoon was mature or immature, he examined material from the caput, corpus and cauda regions of the epididymis. Immature spermatozoa were prominent in the caput region and mature spermatozoa in the cauda region.

Changes in the nucleus The qualitative change in the nuclear material of the bull spermatozoa has been reported by Gledhill (1971). He suggested that the negatively charged phosphate groups of the DNA were masked by new types of proteins which contain more arginine, and were consequently more basic, than the proteins typically bound to DNA in somatic cells. A consequence of the increasing neutralization of the DNA phosphate is that the DNP molecules will become less rigid, attract fewer water molecules, and pack together in a smaller volume. This Gledhill (1971) suggests will explain at least part of the mechanism for the condensation of nuclear chromatin.

Calvin & Bedford (1971) investigated the degree of -S-S- cross linking within mature and immature spermatozoa. They concluded that the major stabilization of the nuclear chromatin of mammalian spermatozoa occurs after spermiation by formation of disulphide cross-links during the final stages of maturation in the duct.

Phospholipids Phospholipids are of major importance in the membrane structure. Ram, bull and boar spermatozoa are known to release phospholipids into the surrounding medium on being exposed to 'cold shock' and freezing.(Darin-Bennett, Poulos & White 1973). Using the

thin-layered chromatographic procedure Grogan, Mayer & Sikes (1966) were able to separate and characterise five major phospholipid fractions in ejaculated and epididymal spermatozoa from the boar. They also suggested the possible presence of two or three minor phospholipid fractions, one of these being phosphatidylserine. The major phospholipids are phosphatidyl choline, phosphatidyl ethanolamine, choline plasmalogen, ethanolamine plasmalogen and sphingomyelin. The results of the investigations by Grogan et al. (1966) indicated that epididymal spermatozoa from all sections of the epididymis contained more of each of the five major phospholipids than ejaculated spermatozoa. Both phosphatidyl choline and phosphatidyl ethanolamine decreased in quantity in an almost parallel manner as the spermatozoa traversed the epididymis. This progressive decrease suggests that the phospholipids may be implicated in the maturation process and may be actively involved in the stabilisation of the membranes of the spermatozoa.

Butler & Roberts (1975) investigated the use of phospholipid compounds as cryo-protective agents for boar spermatozoa. They reported that the resistance of the spermatozoa to cold shock is enhanced by the presence of phosphatidylserine, which appears to be a specific function of this substance since other phospholipids and related compounds were ineffective.

Sperm absorption Jones (1971) observed that spermatozoa with damaged membranes, with a resulting loss of most of the acrosomal contents, were present in all regions of the epididymis. They comprised 5 % of the spermatozoa examined in samples taken from different regions of the duct. As this proportion was constant in each region Jones (1971) concluded that the damaged spermatozoa were neither resorbed nor selectively phagocytosed.

Function of the epididymis in storage of spermatozoa

The cauda region of the epididymis serves as an extragonadal sperm reservoir where the spermatozoa survive for long periods without loss of fertilizing power (Einarsson 1971). The ability of the spermatozoa to survive for long periods in this region is of considerable interest as a possible pointer to the creation of conditions for the maintenance of the viability of spermatozoa in vitro. The factors that are conducive to the survival of the spermatozoa are obscure. It is only feasible to speculate that they are possibly immotile and that their metabolism is in a quiescent state.

One obvious feature of the storage in the cauda is the high concentration of spermatozoa. Einarsson (1971) estimated the concentration of the spermatozoa in the cauda epididymis to be 3.1×10^6 spermatozoa per mm^3, compared to a concentration of 0.27×10^6 spermatozoa per mm^3 in the whole semen ejaculate.

During their passage through the epididymis, the spermatozoa undergo dehydration (Glover 1974); as a consequence of which they have a high percentage dry weight (Table 2). As the water content of the cell could contribute to the initiation of intracellular reactions, the lower water content of the spermatozoa in the epididymis is possibly one of the reasons for the quiescent state and greater survival of caudal spermatozoa than those from whole semen. The dry weight of the sperm cells diminishes when they leave the cauda and come into contact with the seminal vesicle secretions. (Einarsson 1971).

A further factor affecting the storage of the spermatozoa in the epididymis could be the electrolyte concentration of the fluid in the caudal lumen. Crabo (1965) reported a decrease in the concentration of both sodium and chloride ions in samples taken of epididymal fluid from the rete testis to the tail of the epididymis. Einarsson (1971) reported the sodium:potassium ratio was 1.0 in caudal

Summary of the levels of different constituents in the fluid from the cauda epididymis and whole semen in boars Source-Einarsson(1971)

	Cauda epididymis	Whole semen
Sodium (meq/l)	30.2 ± 8.9	123.2 ± 12.7
Potassium (meq/l)	31.3 ± 4.1	16.6 ± 3.2
Chloride (meq/l)	19.3 ± 2.6	103.3 ± 10.2
Inorganic phosphate (mg P/100 ml)	10.23 ± 2.90	1.53 ± 0.41
Calcium (meq/l)	5.65 ± 1.96	5.06 ± 1.22
Magnesium (meq/l)	2.20 ± 0.99	23.68 ± 11.38
Total protein (g/100 ml)	2.77 ± 0.61	2.85 ± 1.05
G.O.T.(K.O.u/ml)	95.4 ± 33.6	11.9 ± 4.7
Osmotic pressure (mOsm/l)	330.7 ± 13.4	309.0 ± 12.4
Dry weight of spermatozoa (%)	25.70- ± 6.05	15.03 ± 2.97
Dry weight of fluid (%)	4.47 ± 1.37	4.16 ± 1.38

Notes: ± figures quoted are standard deviations

G.O.T. - Glutamic-oxaloacetic transaminase

K.O.u/ml - Karmen-Ordell units used to express enzyme activity.

Table 2

fluid from the boar and 7.8 in whole seminal fluid. The exposure of the spermatozoa to an elevated sodium : potassium ratio when they are transferred to seminal fluid during ejaculation would influence the motility and the metabolism of the cell. The low sodium : potassium ratio of the fluid in the cauda epididymis has been ascribed significance for the quiescent state of the spermatozoa by Sørensen & Anderson (1956) and by Salisbury & Lodge (1962). It is also interesting to note that in an investigation into the composition of different buffers for the dilution of semen, Crabo, Brown and Graham (1972) reported that the ionic strength of the diluent was inversely proportional to sperm survival on cold storage and freezing.

The functional abilitity of spermatozoa from the epididymis

The fertilizing ability of spermatozoa from different regions of the epididymis was investigated by Hunter, Holtz & Henfrey (1976). Semen from several regions of the epididymis was surgically inseminated into the oviducts of gilts. The investigation demonstrated that spermatozoa gradually attain the ability for fertilization in the mid and lower corpus regions and become fully functional by the time they reach the proximal cauda region. These findings are in agreement with Holtz & Smidt (1976) who demonstrated that the fertilizing ability of caput spermatozoa was very poor, improved with spermatozoa from the corpus and was very good with samples from the cauda epididymis.

THE STRUCTURE OF THE BOAR SPERMATOZOON

Introduction

Since the invention of the light microscope, mammalian spermatozoa have been studied extensively. With the introduction of the electron microscope, it became possible to examine in detail the finer structures of the cell, particularly the membranes of the head region and the arrangement of the fibres within the tail. This has become feasible with the development of more sophisticated techniques for staining, embedding and sectioning (Watson 1958; Luft 1961 and Jones 1973b).

Several workers have studied the morphology of the boar spermatozoon. These include McKenzie et al (1938); Lasley & Bogart (1944); Holst (1949); Roa & Berry (1949); Hancock (1957, 1966); Bane (1961); Nicander & Bane (1962) and Jones (1971, 1973a).

The head of the spermatozoon

The spermatozoon of the boar closely resembles that of other domestic ungulates (Hancock 1957). The head is approximately 8.5 μ long, and flattened, so that when examined in smears, the broad surface is always presented to the microscopist. In this position the head is approximately twice as long as it is wide. The thickness is about 5 μ, but is not uniform in depth.

The main feature of the head of the spermatozoon is the nucleus, which contains a haploid amount of DNA associated with a arginine-rich histone in a ratio close to 1 : 1 by weight (Monesi 1972). The nucleus is composed of electron dense material with no discernible features, and is enclosed within a typical double membrane, the inner component of which adheres tightly to the nuclear surface.

Surrounding the anterior end of the nucleus is the acrosome. This is a dense, homogeneous structure bounded by a single membrane (Nicander & Bane 1962). The origin of the acrosome is the 'Golgi'

Diagrammatic representation of a mid-sagittal section of the head and neck region of a boar spermatozoan

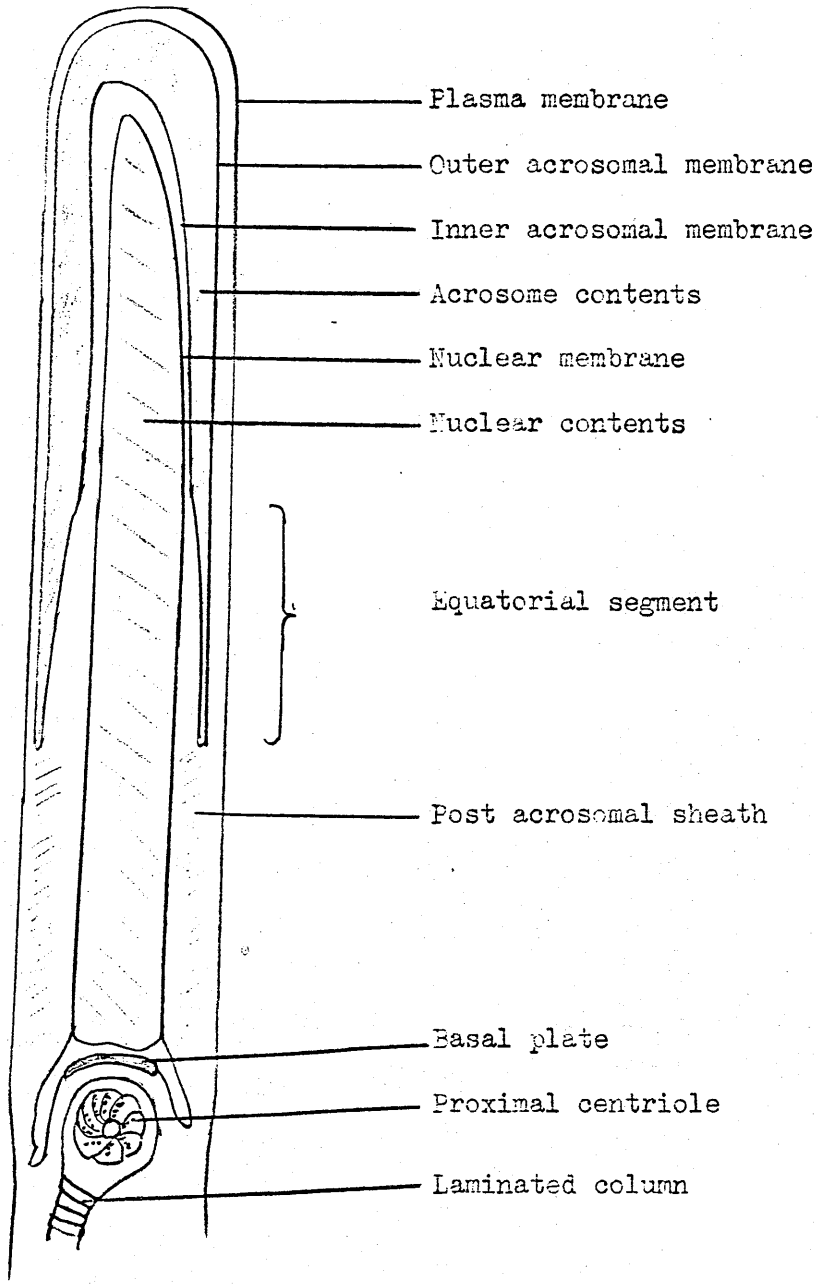


Fig. 5

complex, beginning with several proacrosomal granules, which fuse to form an acrosomal granule, first appearing in the early spermatid and developing into the acrosomal vesicle of the mature sperm.

The acrosome contains PAS-positive and amylase-sensitive polysaccharides and several lysosomal enzymes. These are instrumental in facilitating the penetration of the egg prior to fertilization.

The acrosome tapers at the anterior of the equatorial segment (fig 5). Thus the equatorial segment has a much thinner layer of acrosome than the remaining acrosome-covered area of the nucleus (Nicander & Bane 1962).

Posterior to the acrosome and the equatorial segment is a thin layer of homogeneous material, without a membrane. This constitutes the post-nuclear cap, a prominent feature of spermatozoa examined by light microscopy (Hancock 1957).

Neck region

The neck region of the spermatozoon is the junction of the head with the mid-piece. It is a complicated structure which includes a centriole and a complex organelle by which the axial fibre bundle of the tail is articulated with the head.

Below the posterior boundary of the nucleus, lies the basal plate. This articulates with the capitulum which is supported by two laminated columns whose anterior ends form two implantation plates (fig 6). The columns communicate with nine stout fibres, posterior to which are the nine peripheral fibres of the tail region (Hancock 1966).

In a mid-sagittal section of the neck region (fig 5), the wall of the proximal centriole can be seen. This consists in transverse section of nine elements, each with three tubular structures.

The evagination of the nuclear membrane projects backwards from the posterior border of the head, so the structures of the neck region are covered by the collar of the nuclear membrane, and also by

Diagram of longitudinal section of the neck region of a spermatozoon

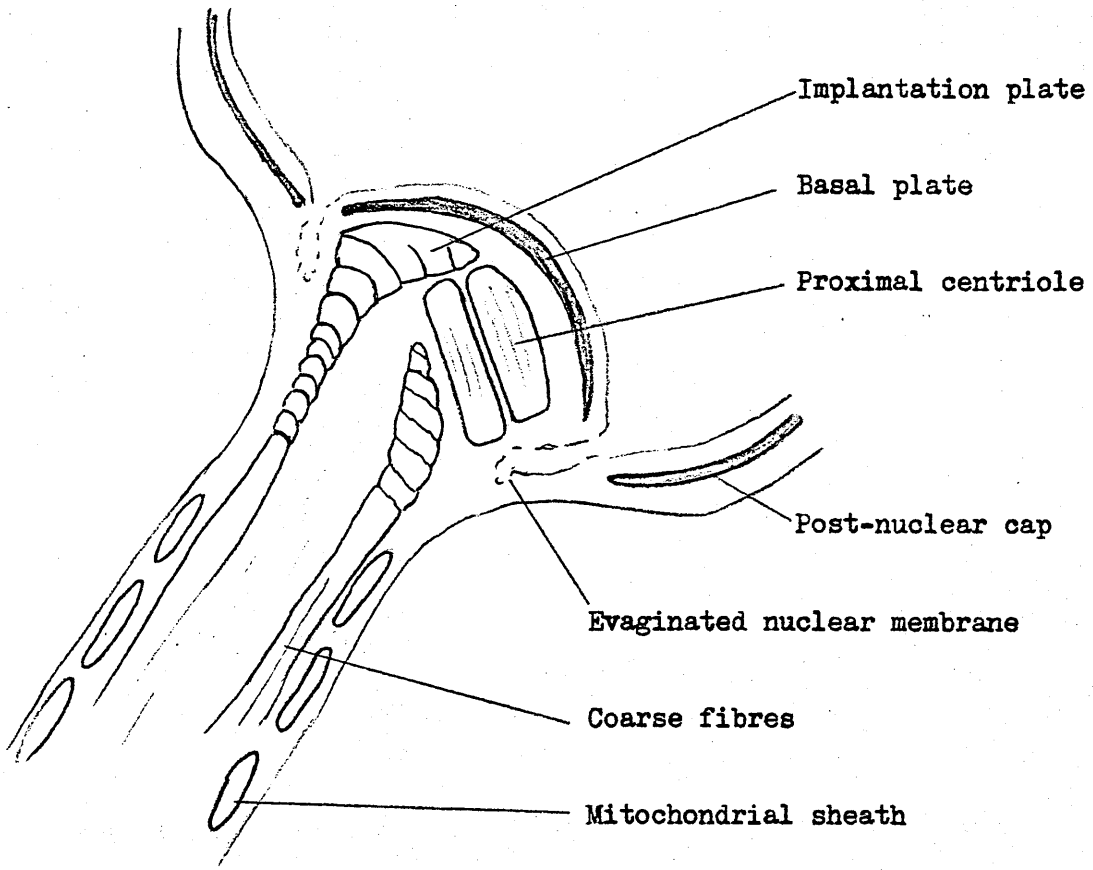


Fig. 6

the overlying plasma membrane (Hancock 1966).

Mid-piece

The mid-piece of the tail region is approximately 10 μ in length, slightly longer than the head (Hancock 1957). It is characterised by the presence of a mitochondrial sheath which is arranged as a double helix surrounding the axial fibrous bundle. The coarse fibres extend from the neck region, through the mid-piece and terminate in the tail-piece at varying distances. They are arranged in a typical 9 + 2 pattern, the two central fibrils being tubular in cross section. The detailed structure and arrangement of the fibres of the boar spermatozoa are given by Nicander & Bane (1962) and for the boar and other species by Hancock (1966).

The mid-piece of the spermatozoon extends from the neck region to a distal terminal ring which forms the posterior boundary.

A common feature of the boar spermatozoon is the cytoplasmic droplet which is found on the mid-piece, generally at the posterior end on the junction of the mid-piece and tail.

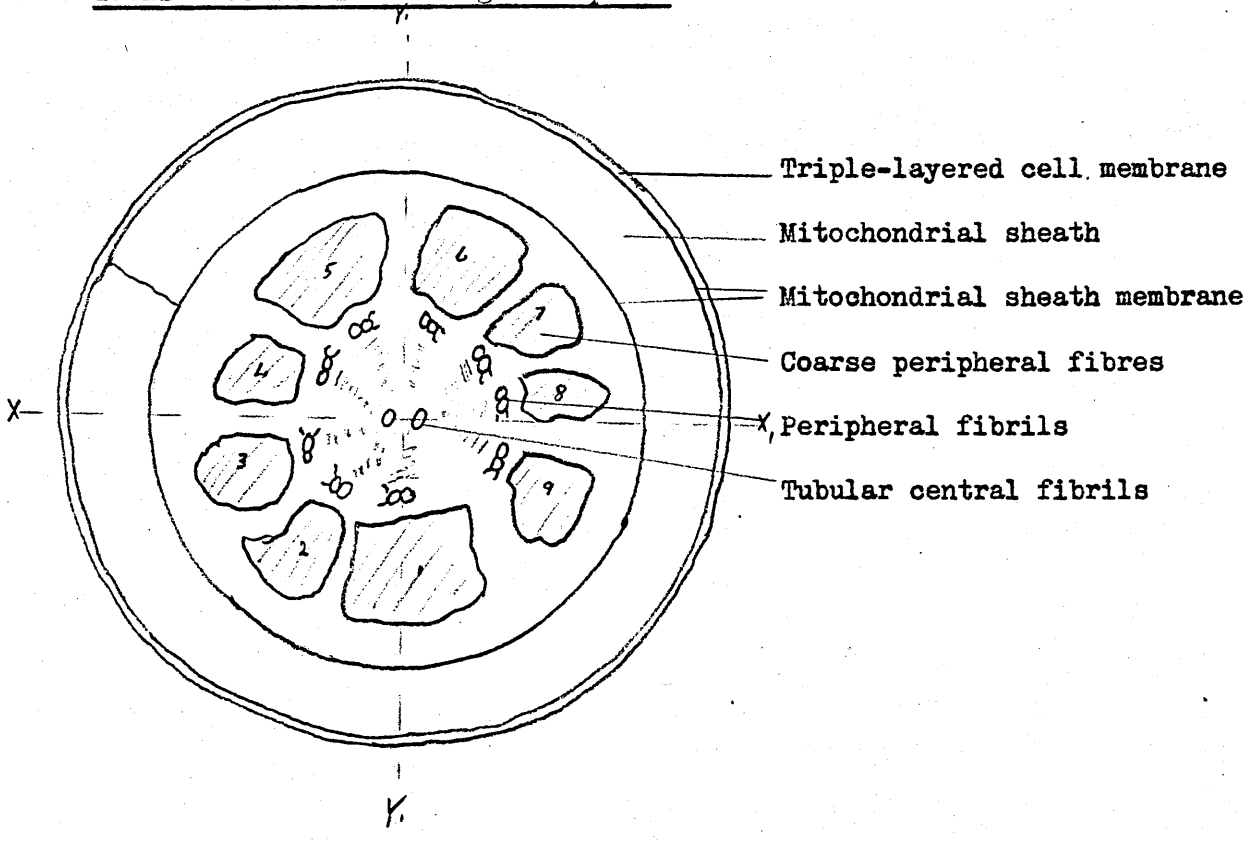
Tail-piece

The tail piece is approximately 30 μ long (Hancock 1957). It extends from the terminal ring of the mid-piece and ends in a short terminal filament. The thickness of the tail gradually decreases along its length.

All the nine coarse fibres continue from the mid-piece into the tail piece where they terminate in a distinct sequence before reaching the terminal filament.

Diagrammatic representation of a transverse section through the mid-piece and tail regions of a boar spermatozoon

Transverse section through mid-piece



Transverse section through tail-piece

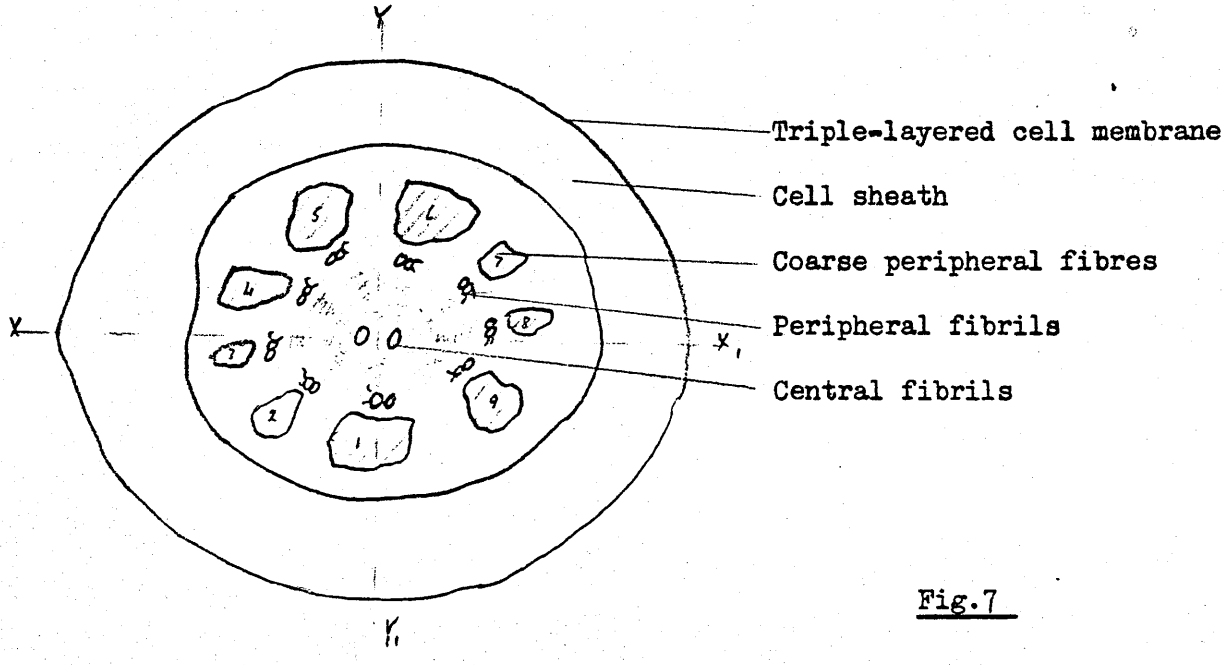


Fig.7

SPERM PRODUCTION RATES

Introduction

A knowledge of sperm production rates is important in order fully to exploit the genetic potential of outstanding boars. This applies particularly to boars that are used for artificial insemination where the frequency of collections can be regulated to meet the demand for semen. Other factors need to be considered apart from obtaining the maximum quantity of semen; these include the quality of the product and the possible effect on the quality of such influences as the level of nutrition of the boars, and the effects of environmental temperature.

The rate of sperm production of the boar has been the subject of several investigations, these include Kennelly (1960); Stevermer, Kovals, Hoekstra & Self (1961); Kenelly & Foote (1961, 1964); Kaplan (1966); Swierstra (1967, 1968, 1971, 1973); Swierstra & Rahnefeld (1967) Wetteman, Wells, Omtvedt, Pope, Turman, Mahoney & Williams (1974). Recent reviews of the methods of measuring sperm production rates have been undertaken by Amann (1970) and Berndtson (1977).

Daily sperm production (DSP) is the total number of sperm produced daily by the testis. Not all sperm that are produced can be accounted for in the ejaculate. Losses may occur through resorption of the spermatozoa from the epididymis. Doubts about resorption have been expressed when histological investigations have failed to show extensive degeneration or phagocytosis of spermatozoa in the epididymis (Lino, Braden & Turnbull 1967). Investigations by Lino et al.(1967) of the number of spermatozoa voided daily in the urine of sexually inactive rams indicate that this is a more satisfactory explanation of the mechanism for the dispersal of surplus spermatozoa in rams and possibly for other species.

The daily sperm output (DSO) is the maximum number of spermatozoa recovered from the ejaculate.

Methods of measuring sperm production and output

Quantitative testicular histology Daily sperm production can be determined by taking tissue samples from the testis and calculating the volume of the round spermatid nuclei present, as the life span of these spermatids within the tissue is known, then the rate of production can be established. This method was developed by Kennelly & Foote (1964) and later modified by Swierstra (1968a).

The tissue samples are taken from three specific locations on the testis, fixed in Allen's fixative for 3 days, sectioned, and stained with Feulgen technique using fast green as a counter-stain. The percentage volume of round spermatid nuclei present on the stained tissue slides are calculated by Chalkley's technique (Chalkley 1943). This involves using a five pointed indicator which is placed in the microscope ocular, the tissue component at the end of each pointer is recorded in a large number of randomly selected fields. After calculating the corrected testis volume, the sperm production rate is determined using the following equation:-

$$DSP = \frac{\text{Corrected testis vol. x vol. \% round spermatid nuclei in testis}}{\text{Av. vol./round spermatid nuclei x life-span round spermatids (days)}}$$

This method of calculating daily sperm production is tedious and time-consuming. It is subject to potential errors from the assumption that no losses occur due to degeneration of the germ cells at later phases of spermatogenesis.

Testicular homogenates This method is similar to the quantitative testicular histology method except that it involves counting the number of spermatid nuclei in a homogenised testis as opposed to a small tissue sample. The number of spermatid nuclei present within the homogenate is divided by a time divisor representing the number of days production these spermatids represent (Amann 1970). The time

divisor may vary according to the species and the strain of boars being investigated.

Cannulation of the testis The establishment of a vas deferens fistula for the collection of the contents produced by the testis of the boar was undertaken by Einarsson (1971), Johnson et al.(1971) and Wierzbowski & Wierzchos (1969). Examination of the material obtained by Einarsson (1971) by the fistula technique from the cauda epididymis indicated that it did not differ appreciably from the material obtained by post mortem from the same area of the epididymis. He did however, report finding changes in spermatozoal morphology in the fistula material, especially in the elevated number of spermatozoa with bent tails.

Other attempts to cannulate the testis have had limited success because of exudative contamination, changes in spermatozoal morphology and difficulty in maintaining an open duct. Berndtson (1977) reported that cannulation may affect resorption of the spermatozoa in the epididymis and it must be precluded in instances in which it is desirable not to impair the reproductive life of the animal.

Depletion of the epididymal reserves The effect of repeated collections of semen at short intervals from the boar, will in theory exhaust the reserves in the epididymis. By frequent regular collections after exhaustion it should be possible to estimate the daily sperm production. Swierstra (1971) collected ejaculates at 72 hour intervals from 12 boars for an 11 week period. The mean number of spermatozoa in the last 10 ejaculates, divided by the collection interval in days indicated a daily sperm output of 15.3 $\pm$ 0.2 x 10^9 sperm. These boars were slaughtered and the daily sperm production was calculated from the testis material by quantitative histology at 16.5 $\pm$ 0.5 x 10^9 sperm. The daily sperm production was also calculated from the epididymal

material by dividing the total sperm reserves in the right and left epididymides by the number of days required for the spermatozoa to pass through the epididymis (10.2 days). Using this technique the daily sperm production was estimated at 15.0 $\pm$ 1.3 x 10^9 sperm. The similarity between these results indicate that frequent collections from the boar can be a useful indication of the rate of daily sperm production. Sperm output and sperm production estimates are similar, suggesting that in sexually active boars, few if any spermatozoa are absorbed from the epididymis or excreted in the urine.

Factors affecting sperm production and output

Age and body weight Puberty of an animal is the age when spermatozoa first appear in the ejaculate, as opposed to sexual maturity when the male first attains maximum daily sperm production (Amann 1970). Examination of the ejaculates of 150 - 180 day old boars by Swierstra (1973) indicated that these boars had attained puberty. Wiggins et al. (1951) found a group of eleven boars to be fertile at 211 days. Niwa & Mizuho (1954) reported that large white boars first ejaculated at 180 - 210 days. Differences in body weight rather than age appears to account for the growth of the reproductive organs and the onset of sexual maturity. (Niwa & Mizuho 1954; Swierstra 1973). As the rate of growth will be affected by the level of nutrition, then the age at which a boar reaches puberty and sexual maturity will be dependant on its food intake.

Storage capacity of cauda epididymis There is a considerable difference between boars in the capacity of the cauda epididymis to act as a semen reservoir. This was demonstrated by Swierstra (1973) who subjected boars to a 72 hourly collection schedule in order to deplete the semen reserves and measured the epididymal contents after slaughter. The variation between boars in their capacity to store spermatozoa may

help explain why some boars are able to sustain a daily output of spermatozoa and others are rapidly depleted.

Testicular weight The production of spermatozoa and the differences in daily production of spermatozoa are largely a function of testicular weight and percentage of seminiferous tubules within the testis (Kennelly 1960). This was to some extent confirmed by Swierstra (1968a) who was able to show a significant correlation between testicular weight and daily sperm production from the combined data of semen collected from Yorkshire and Lacombe boars.

Collection frequency The frequency that boars should be collected from for routine use for artificial insemination remains controversial. Gerrits, Graham & Cole (1962) found that the greatest number of sperm per ejaculate occurred when semen was collected 5 times in twenty days. Polge (1956) recommended two or three collections per week. McKenzie et al (1938) reported successful results when collections were made every 48 hours; this was confirmed by Aamdal (1964) but his results indicated that some boars would have difficulty in maintaining this frequency over long periods.

Swierstra (1973) collected semen from 36, 86 and 139 week old boars at 72 hour intervals. He found that with the older boars, the total, and percentage motile spermatozoa per ejaculate, decreased over the first six collections then tended to stabilise. This was less pronounced in younger boars in which, after an initial decrease in total and percent motile spermatozoa, all semen characteristics except gel. volume showed significant increases as a result of growth of the reproductive system. When the same boars were placed on a 24 hour collection schedule there was a pronounced decrease in the total and percentage motile spermatozoa per ejaculate; after five collections this tended to stabilise again. Swierstra found that more spermatozoa were harvested with a 72 hour ejaculation frequency than during the 24 hour ejaculation frequency.

In previous experiments by Swierstra (1968a, 1971), he reported that sperm output averaged 86 % of total sperm production by the testes (measured by testicular histology) when the semen was collected at 48 hour intervals, sperm output averaged 99 % of total sperm production when the semen was collected at 72 hour intervals. In these experiments, Swierstra was primarily concerned with the total number of spermatozoa harvested, rather than the quality of the spermatozoa.

An investigation into the effect of the ejaculation frequency on the fertility of the spermatozoa was made by Swierstra and Dyck (1976). They reported that a higher pregnancy rate was not regularly obtained with sperm recovered at 24 hour intervals, as compared to 72 hour intervals between collections. They observed a boar - collection frequency interaction, some boars being more fertile with a 24 hour collection rate, while others show a better performance with a 72 hour collection rate.

Environmental temperature The reproductive performance of boars may be affected adversely by exposure to higher ambient temperatures (Signoret & Du Mesnil du Buisson 1968; McNitt & First 1970; Wetteman, Wells, Omtvedt, Pope, Turman, Mahony & Williams 1974; Wetteman, Wells, Omtvedt, Pope, & Turman 1976).

High ambient temperature appears to affect the development of the spermatozoa during the formation process in the testes. Owing to the time taken for the development stages to reach completion and the period of transit through the epididymis, affected spermatozoa will not become apparent in the ejaculate until several weeks after exposure of the animals.

Wettemann et al (1974) exposed six boars to a higher ambient temperature of 34°C to determine the effects of increased temperature on their reproductive performance. When compared to the control

boars maintained at 23°C, differences became apparent in the semen ejaculate with the appearance of spermatozoa with 'aged' acrosomes from the treated boars. After 6 weeks, sperm motility was 20 % less in semen from the treated boars. There was a marked decrease in fertility, demonstrated by the number of embryos present in inseminated sows. The boars were slaughtered after 90 days; examination of the reproductive organs showed that the weights of the testes and epididymides were similar for the control and treated boars, but that total testicular sperm and epididymal sperm were reduced by 50 % in the heat treated boars. The depletion of spermatozoa in the testis and the associated loss of motility and ferility suggests that the exposure to high ambient temperature, may affect some stage of spermatogenesis.

Work undertaken by Brooks (1973) on rats, and by Waites and Setchell (1969) on rams, demonstrate the inability of these animals to maintain the normal temperature differential between abdominal cavity, testes and epididymides when exposed to high ambient temperatures. The results of these experiments, added to the reports by Signoret *et al.* (1968), McNitt *et al.* (1970) and Wetteman *et al.* (1974) working with pigs, all indicate that high ambient temperature is detrimental to the production of spermatozoa by the boar.

There is no evidence of hypothermia affecting semen production by boars. Swierstra (1970) exposed boars to a mean daily temperature of -15 to -20°C for a 15 week experimental period. Exposure to cold did not interfere with testicular development, production of sperm or seminal quality.

THE COLLECTION AND EXAMINATION OF BOAR SEMEN

Introduction

The collection of semen from the boar and the examination of the semen-ejaculate to determine the 'quality' and concentration of the spermatozoa is an essential part of the routine of an artificial insemination centre. It is important to be able to extract semen from the boar on a regular basis and to employ some method of evaluation to ensure a satisfactory standard before dilution and storage.

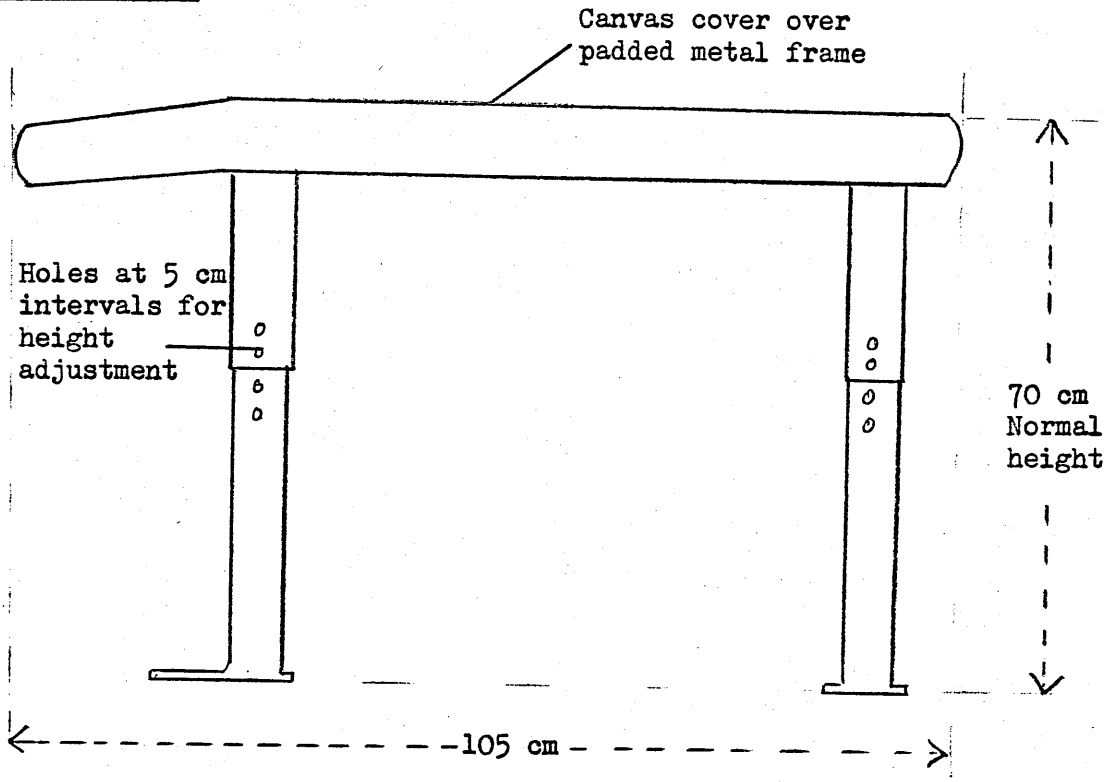
Collection of semen

'The training of boars to mount a dummy sow and to ejaculate usable semen on a regular basis does not present many difficulties' (MacPherson 1970). The basic requirement is a dummy sow which should be strongly built, preferably of metal, and well padded so the boar can rest on it comfortably. The padding should be enclosed beneath a strong canvas cover which fits neatly over the padding with no loose ends protruding to distract the boar's attention. The whole structure should be rigid and the height adjustable to cater for both large and small boars (Fig 8).

Training the boars to mount the dummy sow is achieved primarily by olfactory stimuli. It is useful if the training of boars takes place on the dummy sow after routine collections from working boars, so that the odour from the previous collections is still present. Alternatively, the dummy sow can be sprinkled with semen from another boar. A further means of encouraging a boar to mount is by visual stimuli; A collection from a working boar adjacent to the pens holding the training boars takes place, so that the training boars can observe the boar using the dummy sow. This will cause sexual arousal (Campbell & Lingham 1965). Other methods include massaging the prepuce, slapping the canvas of the dummy sow to attract the boar's attention, blowing in the face of the boar and

Diagram of a dummy sow used for collection of semen from the boar

Side elevation



End elevation

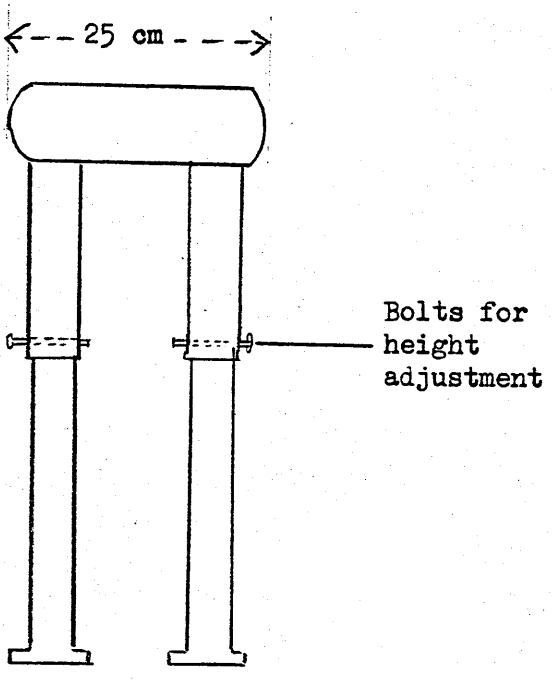


Fig 8

if necessary the operator vacating the collection area so that the boar is left alone with the dummy sow for a short period.

Differences in libido have been found to depend on breed and age of boar, younger boars being easier to train than older ones (Reed 1966). If a complete lack of interest in the dummy sow occurs, then an ovariectomised gilt (maiden female pig) treated with stilboestrol dipropioate to bring it on 'heat', can be used in the collection area to stimulate the boar(Hancock 1959). Generally, boars that are difficult to train are the slowest to mount the dummy sow in regular use (Reed 1966).

Once mounted, the boar commences thrusting movements. The penis, which under natural service would become fixed in the cervical folds of the sow, must now be fixed by other means before the boar will ejaculate.

Several workers have described methods of fixation. The penis can be directed into a thin walled cone-shaped rubber tube, the operator grips by hand the glans penis through the tube, using the fingers to simulate the sows cervical ridges. The penis becomes locked and ejaculation proceeds (McKenzie 1931; Ito, Niwa, Kudo & Muzuko 1948).

Polge (1956) describes a modification of the bull artificial vagina (A.V.) which can be used for boars. This is basically a cylindrical rubber casing with a thin rubber lining. The space between the casing and the lining is filled with warm water which is emptied prior to the collection and a pump attached to fill the space with air. It is possible, by means of non-return valves, to cause the internal lining of the artificial vagina to pulsate when the penis has become fixed.

Melrose & O'Hagan (1959) introduced a metal spiral which is attached to one end of the artificial vagina. A central rubber

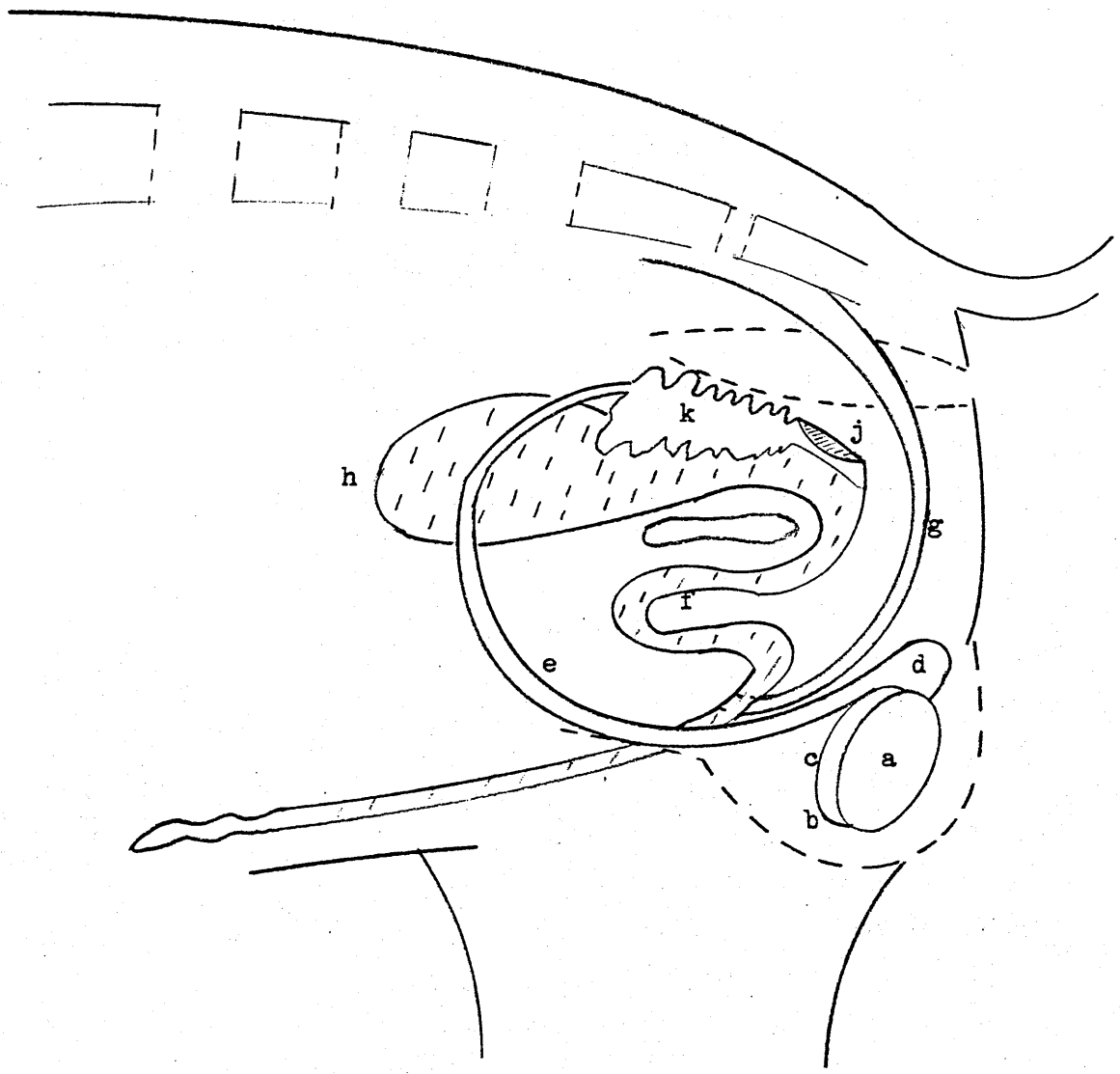
cone extends through the full length of the casing into the rubber covered spiral. The boar thrusts his penis through the artificial vagina and locks in the ridges of the spiral.

The present accepted method of collecting semen is by gripping the glans penis between the fingers, using a thin rubber glove.(Hancock & Hovall 1959). A comparison has been made of the 'gloved hand' technique and the use of an artificial vagina by King & Macpherson (1973), who concluded that 'the advantage of using the gloved hand is the simplicity, low cost and the elimination of the need for cleaning and sterilizing of equipment. This method provides adequate tactile stimulus to achieve optimum sperm output and is satisfactory for the routine collections of semen'.

Park, Melrose, Stewart & O'Hagan (1964) reported a marked variation in the numbers of bacteria present in the semen taken from the same boars on different occasions. The bacterial contamination comes primarily from the preputial diverticulum (Koppang & Filspeth 1958), the actual ejaculate from the tip of the penis having a low bacterial count. To reduce the number of bacteria present in the semen collection, the first part of the ejaculate i.e. the pre-sperm fraction, should be discarded and an absorbent sponge diaphragm fitted between the artificial vagina or the operator's hand and the prepuce, to absorb any flow of preputial fluid down the penis which could contaminate the semen (Bonadonna, Roychouda, Nelli & Pedron 1972).

The process of ejaculation is prolonged and may take from 3 - 20 minutes (Polge 1956). The semen is emitted in three distinct fractions. 1) Pre sperm - an almost clear fluid containing jelly like material. 2) Sperm rich fraction - a thick white coloured fluid which has a high concentration of sperm. 3) A clear post sperm fraction. With the post sperm fraction, there is produced a large quantity of gelatinous material, in tapioca-like lumps which swell

GENITALIA OF THE BOAR



a - Testis
b - Caput epididymis
c - Corpus epididymis
d - Cauda epididymis
e - Vas deferens
f - Sigmoid flexure of penis
g - Refractor penis muscle
h - Urinary bladder
j - Bulbo urethral gland
k - Seminal vesicle

Fig. 9

on exposure to air. The function of this material is not clear, but it could serve as a plug to prevent the back-flow of semen in the female tract after insemination.

Some boars will produce a second ejaculate if allowed to remain on the dummy sow. The second ejaculate follows the same pattern as the first.

The receptacle for the collection of semen should be pre-warmed to 30°C and insulated to prevent cold shock. A 250 ml reagent bottle held in a thermos flask is ideal. The separation of the gelatinous material is achieved by placing a polypropylene funnel, lined with either a milk filter or absorbent gauze in the neck of the reagent bottle to filter the semen ejaculate.

Examination of Semen

The various laboratory methods of examining semen after collection, will not give a precise estimate of the boar's potential fertility, but will give some indication of it. (Reed 1976).

The following tests are carried out routinely by artificial insemination centres :

Motility A sample, taken from the ejaculate immediately following the collection, is placed on a heated microscope slide at 37°C, and examined under the light microscope at x 80 magnification. A good sample will show individual movement of all the spermatozoa, the more concentrated samples will display a characteristic 'wave motion'. Walton (1952) made a study of 'wave motion' in cattle semen. He explained the cause of this motion as being spermatozoa which face in one direction forming a stream by their movement in that direction, with the result that various streams, flowing in different directions at random, induce a certain amount of flow orientation. The speed of movement of such streams of spermatozoa creates the appearance of light and dark areas, or waves, due to the variation in the amount

of scatter of light from the different streams of spermatozoa.

Poor samples of semen display a sluggish movement. When large numbers of dead spermatozoa are present, they will be inclined to stick together. This agglutination of the spermatozoa is a characteristic of poor semen quality and is referred to as 'clumping'.

It is important when making motility observations that the temperature of the microscope slide is controlled by using a 'warm stage'. Variation in the temperature of the slide affects the rate of movement of the spermatozoa. A cold slide will markedly reduce the degree of motility.

Volume The volume of semen collected from an ejaculation can vary from 50 to 500 ml depending on the volume of clear seminal fluid taken during the collection. The larger volumes collected usually contain the same number of spermatozoa that have been diluted to a greater extent. For routine use on an artificial insemination centre, where the semen is diluted in a buffer solution, there is no advantage in collecting the clear fraction of the ejaculate.

Density The concentration of the spermatozoa in the semen collection may be measured using a haemocytometer counting chamber.

As the opacity of the semen is proportional to the spermatozoal concentration, most artificial insemination centres now use a photo-electric colorimeter or absorptiometer to measure semen density. The calibration and use of a standard 'EEL' colorimeter for the estimation of the number of spermatozoa in bull semen is given by Cox & Melrose (1953). The same principles apply when calibrating the colorimeter for boar semen, apart from the dilution rates which require adjustment, owing to the more dilute semen from the boar. Cox & Melrose (1953) recommended occasional check calibrations and advised the regular use of a standard opacity tube to detect any errors due to light variations.

The density of spermatozoa in an ejaculate can vary from 1×10^8 to 15×10^8 spermatozoa per ml (Reed 1969). The density variation of the semen does not appear to have any relation to the potential fertility of the semen samples for use in routine inseminator work. However, the measurement of spermatozoal density is required, in order that the spermatozoal concentration in the diluted semen remains above the threshold level necessary for optimum fertility (Melrose 1962).

Staining techniques for the detection of spermatozoal abnormalities

The microscopical examination of stained spermatozoa to detect the presence of abnormal spermatozoa is a further aid to the determination of semen quality.

Several stains are suitable for this purpose. A review of the staining techniques for mammalian spermatozoa was presented by Hackett and Macpherson (1965), who outlined the preparation procedures and dyes used for morpological staining. These include rose bengal, methyl-violet and Indian ink, for differential staining - eosin/fast blue, eosin/fast green, eosin/aniline blue, revector soluble blue and congo red/nigrosin. The use of fluorescent dyes and Feulgen DNA stain are also reviewed.

Other techniques used for staining boar spermatozoa are Giemsa (Hancock 1952), Heidenhain's iron hoematoxylin (Pantin 1946), Periodic acid-Schiff (PAS) method (Pearse 1953) and silver impregnation (Hancock & Trevan 1957).

The decision on which staining technique to use, is dependant on the morphological details being examined. The limits of the outer acrosome are most clearly shown in spermatozoa stained by the PAS and Giemsa methods. The equatorial segment is most prominent in Giemsa-stained spermatozoa, whilst the post-nuclear cap is shown clearly in live spermatozoa impregnated with silver (Hancock 1955).

For the routine examination of spermatozoa on an artificial insemination centre, the most popular stain is nigrosin/eosin (Campbell, Hancock and Rothschild 1953), primarily because this stain is inexpensive, easy to prepare, and simple to use. (Appendix 6).

Live and dead counts The assessment of live/dead spermatozoa by the differential staining technique was first used by Lasley, Easley & McKenzie (1942).

Live and dead cells can be discriminated on the basis of their permeability to eosin, using a suitable background stain such as opal blue or nigrosin. Dead cells are permeable to the eosin stain possibly as a result of membrane changes (Foote 1969).

The accuracy of the live/dead count for boar spermatozoa is questionable. Variations occur due to the period of exposure of the spermatozoa to the stain, the selection of the fields on the slide examined, and the effects of the clumping together of the dead spermatozoa. Possibly the greatest variation can be attributed to the difference of interpretation caused by the presence of many partially stained cells and the examiners interpretation of stained and unstained (Campbell, Dott & Glover 1956).

Other methods of the assessment of semen Investigations of methods to determine semen quality by laboratory techniques have been undertaken by several workers, particularly on bovine semen.

Umbreit, Burris & Stauffer (1964) described a method of measuring oxygen consumption of diluted samples using a Warburg constant volume respirometer. Melrose and Terner (1952, 1953) reported that the quality of bovine semen samples could be graded according to the oxygen consumption, after the addition of pyruvate and pyruvate plus 2:4 dinitrophenol, to washed spermatozoa.

Measurement of the pH of bovine semen at the time of collection was shown not to have any practical value (Anderson 1945; Laing 1945;

Reid, Ward & Salisbury 1948). although Fiser (1952) reported that the fall in pH on incubation of semen gave an indication of semen quality.

Relationship between semen evaluation methods and fertility

Singleton and Shelby (1972) evaluated the variation of semen characteristics i.e. oxygen consumption, pH, motility and spermatozoal abnormalities between boars, between ejaculates within boars, and their relationship to fecundity in the gilt. They reported finding significant differences between boars for most of the semen characteristics measured. They concluded that the variation between ejaculates within the same boar indicates a possible source of error when using only one, or a few ejaculates, to predict the future fertility of boars.

Linford, Glover, Bishop & Stewart (1976) studied the relationship between laboratory semen evaluation tests and fertility in the bull. These tests included motility assessments, morphology examination and pH measurements. The results of their investigations reveal the inadequacy of laboratory tests when used as a method of predicting the fertility of semen samples. However, they do show that limits may be set, outside which, poor semen samples could be discarded.

With boar semen, the initial motility estimation and other laboratory tests can be used to detect gross differences in semen quality. They have a limited value in detecting small fertility differences. In practice, the procedure is to take into consideration several parameters including laboratory tests and fertility results from previous collections, before deciding that an ejaculate should be discarded.

EXPERIMENT 1

The Relationship of Morphological Abnormalities in Boar Spermatozoa to Conception Rate and Litter Size

Introduction Several workers have studied the incidence of specific morphological classes of boar spermatozoa, including McKenzie et al (1938), Hancock (1959), and Singleton & Shelby (1972).

This investigation was undertaken 1) to compare the incidence of morphologically abnormal spermatozoa in semen of fertile boars to the observations of previous workers. 2) To compare the incidence of abnormal spermatozoa in semen with conception rates and litter size over a period of one year. 3) To examine the possibility of a seasonal variation of the incidence of abnormal spermatozoa.

Materials and Methods Five pure bred Large White (LW) and five pure bred Landrace (LR) boars from the Meat and Livestock Commission's Pig Breeding Centre, Selby were used. The boars were collected from each week, the semen was diluted in EDTA diluent and used for routine artificial insemination.

A semen sample was taken from an ejaculate from each boar for examination, once per month over a period of twelve months. The samples were stained as soon as possible after collection using nigrosin eosin stain (Campbell et al.1953). Two smears were prepared from each sample, and counts made of a total of 100 spermatozoa from each smear.

The morphological abnormalities recorded included - detached heads, malformed heads, damaged acrosome caps, coiled tails, bent tails and cytoplasmic droplets. (Fig10). The cytoplasmic droplets were differentiated into proximal, middle, and distal, according to their location on the mid-piece of the tail.

For comparison with conception rates, the abnormalities were summarised into three groups - 1) total number of abnormal spermatozoa

2) total number of abnormal spermatozoa excluding cytoplasmic droplets, and 3) total number of cytoplasmic droplets.

The related figures for conception rates and litter size were obtained by using the boars for artificial insemination over the same period.

Correlation coefficients between the groups of spermatozoal abnormalities observed, and the conception rate and litter size, were analysed, using Kendall's Coefficient of rank correlation (Kendall 1962).

Results and discussion The results of this and three previous surveys of the morphological classes of abnormal spermatozoa in boar semen are summarised in Table 3.

Hancock (1959) remarked on the difficulty of comparing results, because of the different methods of classification used, and because of differences in the presentation of the results. The reclassification of the results of McKenzie et al. by Hancock, of neck beads was repeated, and the term 'droplets' is now used instead of 'beads'.

Both Hancock (1959) and Singleton et al. (1972), refer to malformed middle-pieces, the incidence of which they recorded as 2.7 % and 6.0 % respectively. In this investigation no similar abnormality was observed, possibly because of the different staining procedure. Several disparities exist in the illustrations by Hancock, on the distinguishing features of the morphological classes. In particular, the illustration of the malformed mid-piece is at variance with the photomicrograph depicting the same abnormality. Singleton et al. (1972) have no illustrations of the range of abnormal spermatozoal classes under discussion.

The frequencies of cytoplasmic droplets recorded by Hancock (1959) (Table 3) is considerably higher than in the other investigations.

This high rate of droplets, is explained by Hancock, as being due to collection techniques. "As the droplet is lost from the spermatozoa, when the spermatozoa come into contact with the accessory secretions, by collecting the sperm rich fraction only, mixture with the secretions is prevented". Hancock also states, "the proportion of droplets which undergo disintergration will increase with time after ejaculation, so the interval between collection and examination will be a further source of error, unless the spermatozoa undergo fixation".

The frequency of the collection of semen from the boars prior to use for his investigation, could also account for the higher incidence of droplets reported by Hancock.

A comparison was made of the mean percentage of morphological abnormal spermatozoa, and conception rates and litter size, during a twelve month period (Table 4). A statistical analysis was undertaken to see if there is any correlation of percentage total abnormal sperm--atozoa, with conception rate and litter size; percentage total abnormal spermatozoa excluding cytoplasmic droplets, with conception rate and litter size; and percentage total cytoplasmic droplets, with conception rate and litter size. The correlations were not significant in any of these results. This is possibly because of the comparison of only small variations of morphological abnormalities with small variations in conception rate and litter size.

These results are contrary to the observations of Singleton et el. (1972) who obtained a significant correlation ($P < .05$) between the incidence of normal spermatozoa and fecundity. This would have been reflected on Table 4 with an inverse relationship between the total abnormal spermatozoa and conception rate.

The introduction of several infertile boars, or boars whose semen had high levels of abnormalities, into this experiment would make the results more interesting. It is however, impractical to

use boars with suspect fertility for the commercial artificial insemination of pigs.

The investigation into the variation of the levels of spermatozoal abnormalities during the periods - May to October, and November to April, are given in Tables 5 & 6. They are summarised in Table 7. The division of the experiment into two 6 monthly periods, based on the warmest and the coldest months, was to investigate the possible seasonal variation in the number of abnormal spermatozoa. These could be a possible contributing factor to the lower conception rates that are atributed to hot weather conditions (Wetteman et al. 1974).

The results (Table 7) are remarkably consistent between the levels of spermatozoal abnormalities during the two 6 month periods. Also there is no significant variation between the conception rates and average litter size. A possible explanation for the lack of variation may be in the housing and management of the boars. In particular, the better insulation and ventilation of buildings, enables a controlled environment that should eliminate seasonal variations in spermatozoal production (Signoret et al. 1968).

The mean percentage of nine morphological classes of spermatozoa in the ejaculated semen of ten fertile boars over a twelve month period

	Detached heads	Malformed heads	Abaxial heads	Damaged acrosome cap	Cytoplasmic droplets			Coiled tails	Bent tails
					Proximal	Middle	Distal		
Mean	0.13	0.40	0.03	0.13	1.67	0.09	5.05	0.16	2.10
sd	0.49	0.66	0.22	0.41	1.87	0.25	6.09	0.41	4.19
Other workers investigations									
McKenzie et al (1938)	0.9	0.7	-	-	2.4	-	4.0	-	6.2
Hancock (1959)	0.3	3.0	-	-	11.8	-	17.8	0.09	4.5
Singleton et al (1972)	-	6.0	-	-	-	-	2.0	1.0	9.0

Table 3

The mean percentage of morphological abnormal spermatozoa and conception rates of ten fertile boars during the 12 month period January - December

Boar	Total abnorm. spermatozoa	sd	Total exclud. cyto.droplets	sd	Total cyto. droplets	sd	No.sows insem/preg	Conception rate %	Av. litter size
LW 1	13.8	6.9	6.6	6.3	7.2	3.1	167/131	78	10.0
LW 2	3.9	1.6	0.9	1.3	3.0	1.5	524/415	79	10.9
LW 3	20.2	10.75	2.0	0.9	18.2	11.6	154/113	73	9.8
LW 4	6.2	2.5	2.0	1.2	4.2	2.1	104/70	67	10.3
LW 5	5.7	4.5	1.2	1.5	4.5	3.3	438/352	80	10.2
LR 1	14.5	8.7	3.8	8.2	10.7	9.3	194/155	80	10.2
LR 2	4.6	1.9	1.5	1.5	3.1	2.3	92/71	77	11.3
LR 3	9.3	3.8	3.1	2.6	6.2	2.9	391/294	75	10.6
LR 4	3.4	2.0	1.6	1.8	1.8	0.1	234/155	66	9.8
LR 5	16.0	5.5	6.8	3.6	9.2	4.9	311/235	76	10.5

Table 4

The mean percentage of morphological abnormal spermatozoa and conception rates of ten fertile boars during the six month period May - October

Boar	Total abnorm. spermatozoa	sd	Total exclud. cyto.droplets	sd	Total cyto. droplets	sd	No.sows insem/preg.	Conception rate %	Av.litter size
LW 1	16.8	6.5	10.4	6.7	6.4	0.5	26/21	81	9.8
LW 2	3.3	1.8	1.3	1.8	2.0	1.1	295/243	82	11.1
LW 3	19.6	7.8	2.3	0.8	17.5	8.5	75/53	71	9.5
LW 4	7.7	2.4	1.8	1.6	5.9	1.6	30/17	57	9.8
LW 5	6.5	5.9	1.0	2.0	5.5	3.9	138/107	78	10.3
LR 1	14.6	9.2	1.0	0.9	13.6	8.7	21/17	81	10.6
LR 2	4.3	1.9	1.4	1.0	2.9	1.2	87/66	76	11.4
LR 3	11.1	4.1	4.8	2.5	6.3	3.4	270/214	79	10.7
LR 4	2.5	1.0	1.0	1.1	1.5	0.5	187/122	65	9.8
LR 5	14.7	3.7	7.7	4.4	7.0	3.6	193/155	80	10.6

Table 5

The mean percentage of morphological abnormal spermatozoa and conception rates of ten fertile boars during the six month period November - April

Boar	Total abnorm. spermatozoa	sd	Total exclud. cyto.droplets	sd	Total cyto. droplets	sd	No. sows insem/preg.	Conception rate %	Av.litter size
LW 1	10.8	6.2	2.8	2.6	8.0	4.3	141/110	78	10.0
LW 2	4.4	1.4	0.5	0.5	3.9	1.3	229/173	75	10.6
LW 3	21.0	13.8	1.7	1.0	19.3	14.9	79/60	76	10.0
LW 4	4.5	1.2	2.1	0.7	2.4	1.0	74/53	72	10.5
LW 5	4.9	2.9	1.4	0.9	3.5	2.6	300/245	82	10.2
LR 1	14.4	9.1	6.6	12.7	7.8	4.4	194/155	80	10.2
LR 2	4.8	2.0	1.5	2.0	3.3	3.2	5/5	100	10.6
LR 3	7.5	2.8	1.3	1.0	6.2	2.3	121/80	66	10.3
LR 4	4.2	2.4	2.2	2.3	2.0	1.3	47/33	70	9.9
LR 5	17.3	7.0	5.9	2.6	11.4	5.2	118/80	68	10.4

Table 6

Summary of the mean percentage of abnormal spermatozoa and conception rates of ten fertile boars, over two 6 month periods. May - October and November - April

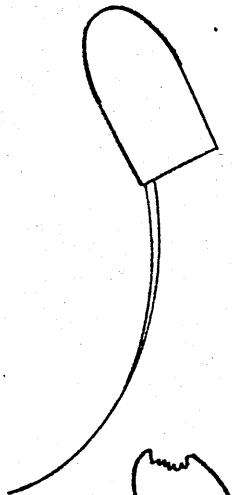
	Total abnorm. spermatozoa	Total exclud. cyto.droplets	Total cyto. droplets	No. sows insem/preg.	Conception rate %	Av.litter size
May to October						
Mean (± sd)	10.1 ± 7.5	3.3 ± 4.1	6.8 ± 6.3	1322/1015	77 %	10.1
November to April						
Mean (± sd)	9.4 ± 6.3	2.6 ± 4.4	6.8 ± 7.8	1308/993	76 %	10.3

Table 7

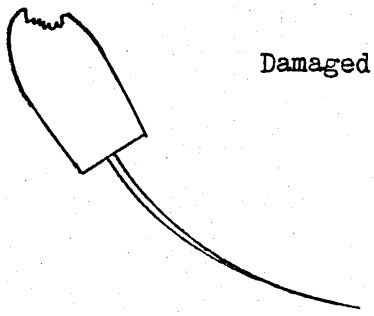
CLASSIFICATION OF SPERMATOZOAL DEFECTS

Detached heads - head completely detached from the mid-piece and tail.

Malformed head - any deviation from the normal shaped head



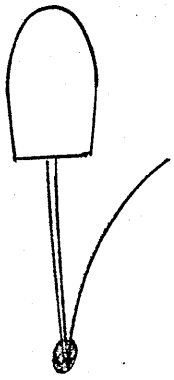
Abaxial head - mid- piece of the spermatozoa not attached to the posterior end of the head at its central point



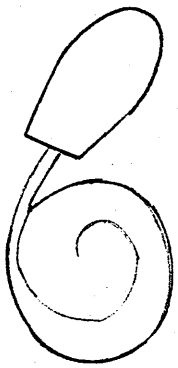
Damaged acrosome cap - any indentation in the apical ridge of the spermatozoal head

Fig 10

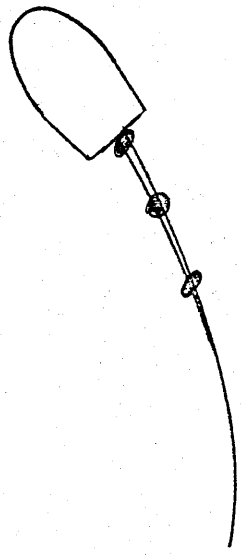
CLASSIFICATION OF SPERMATOZOAL DEFECTS



Bent tail - tail bent back at an acute angle



Coiled tail - tail is coiled like a watch spring



Cytoplasmic droplets - a single droplet situated in one of three positions along the mid-piece

Proximal

Middle

Distal

Fig 10 (cont.)

SECTION II - THE PRESERVATION OF BOAR SPERMATOZOA

Introduction

Spermatozoa will retain their ability to fertilize for several hours after ejaculation, providing that they are maintained under suitable conditions of hygiene and temperature. The ejaculate may be divided into several portions of not less than 50 ml, each of these being used as one insemination dose.

This is the basic use of semen for artificial insemination and can be used 'on the farm' to extend the output from a single boar, thereby obtaining more progeny and a wider dispersal of the genetic material from one animal.

When the situation arises that semen needs to be transported from farm to farm, or stored to await the female reaching the correct stage of oestrus, more sophisticated techniques are employed to prolong the viability of the spermatozoa. These techniques basically involve the dilution of the semen ejaculate in a diluent, then storing at the correct temperature until required.

With the increase in demand of boar semen for artificial insemination, a basic knowledge of the diluents for the storage of semen may be assumed to be an essential requirement for the development of preservation methods to meet this demand.

In Section II, the functions of the diluent are examined, also the various advantages and disadvantages of using 'fresh' as opposed to frozen semen. The several diluents used for the storage of 'fresh' semen are studied and an examination is made of the various procedures involved with the freezing of boar semen.

THE FUNCTIONS OF DILUENTS

Introduction

The diluent is a synthetic medium capable of preserving the integrity of the spermatozoal cells. It is sometimes refered to as a 'buffer' or an 'extender' solution.

The functions of the diluent are 1) to increase the volume of the semen ejaculate so that it can be used for multiple inseminations. 2) To provide a 'buffer' to prevent harmful shifts in the pH as lactic acid is formed. 3) To maintain a proper osmotic pressure and electrolyte balance. 4) To provide nutrients as a source of energy. 5) To inhibit bacterial growth.

When the spermatozoa are stored at low temperatures, the diluent should protect against the harmful effects of cooling, freezing and thawing.

Effect of volume Early experiments undertaken by Milovanov (1934) on the artificial insemination of pigs with undiluted fresh semen, indicated that the volume of the insemination was an important factor in achieving the highest percentage fertilization. He suggested that a large volume of fluid was required in this species to fill the uterine horns and permit the transport of spermatozoa to the fallopian tubes. This was supported by Crabo (personal communication) who recommended a minimum insemination volume of 50 ml; he proposed that it is the physical volume of fluid inseminated in the female that induces uterine contractions. A large inseminate volume was also considered to be necessary by Rodolfo (1934, Du Mesnil du Buisson and Dauzier (1955) Polge (1956), Bower (1974).

Pursel and Johnson (1976b), working on frozen boar spermatozoa, studied the effect of the volume of semen and the number of inseminations required to achieve pregnancy in the sow. Their data indicates a higher pregnancy rate for an insemination volume of 100 ml, compared

to an insemination volume of 50 ml. (71 % vs 50 %).

Other workers who have investigated the effect of insemination volume on the fertility of pigs, include - Stratham & Self (1960) Hancock & Hovell (1961) Baker, Dziuk & Norton (1968).

pH and Oxygen consumption One of the functions of a diluent is that it is required to act as a buffer to maintain a suitable pH during the storage of semen in vitro. This is especially important when the spermatozoal concentration in the diluent, and the storage temperatures, are high enough to permit autotoxication through the acidic products of metabolism (Foote 1969).

Hancock (1959) measured the pH of semen after ejaculation. The mean pH of thirty four first ejaculates was 7.22 (range 6.85 - 7.90) He found that there was a marked increase in pH with time after collections when semen was stored in open vessels. This did not occur when semen was stored in filled stoppered vessels.

Boar spermatozoa are comparitively ineffective 'anaerobes'. The rate at which they metabolise anaerobically, fructose to lactic acid, is much lower than that encountered in the bull and ram (Aalbers, Mann & Polge 1961). Aerobically, boar spermatozoa convert fructose to lactic acid at the same or an even lower rate, yet their motility is very high. This is due to the ability of boar spermatozoa to oxidize lactic acid.

Aalbers et al. (1961) observed that the rate of oxygen consumption by boar semen appears to increase rather than decrease as a result of storage in vitro, despite the decline of spermatozoal motility. The mechanism involved in this phenomenon is not understood. A possible explanation is that the enhancement of the oxidative ability is the outcome of increased cellular permeability due to spermatozoal senesence.

Nevo, Polge & Frederick (1970) observed that when the

ejaculate was collected and maintained under anaerobic conditions, fructolysis, acid production and motility were zero, and the spermatozoa could not be reactivated by prolonged aeration. However, spermatozoa aerobically incubated after ejaculation maintained good motility, and fructolysis and acid production continued for a relatively long time. This did not decline when the spermatozoa where subsequently put under anaerobic conditions. Nevo et al. (1970) concluded that spermatozoa apparently required a period of aerobic incubation in order to maintain full metabolic and physical activity.

The atmospheric level of carbon dioxide that will promote optimum uptake of oxygen by diluted boar spermatozoa was investigated by Sandford & King (1972). They reported that a level of 1 % of carbon dioxide would be sufficient for maintaining a high rate of oxygen uptake by diluted boar spermatozoa, but this would seem to depend on the diluent used. Their results also suggest that it is not necessary to maintain semen pH at its initial level to create conditions for optimum oxygen uptake.

An earlier report by King and Macpherson (1966), proposed that storing boar semen at a pH other than optimum for metabolic activity, would reduce biochemical functions, and would perhaps facilitate the preservation of the fertilizing capacity of the cells.

Poole, Foley & Plotka (1973) reported that the age of boar influenced the buffering capacity and the buffering structure of semen. Boar differences were observed for acidic, alkaline and total buffering capacities.

Osmotic pressure and electrolytes Milovanov (1934) and Selivanova (1934) advanced the theory that electrolytes were harmful to boar spermatozoa and destroyed the 'lipid capsule' surrounding the head. Investigations into the effect of some buffers on storage and freezing indicate the the most promising are those containing relatively few electrolytes. (Crabo, Brown & Graham 1972).

The osmotic pressure of boar seminal fluid is similar to other physiological fluids. Hancock (1959), using a Hortvet cryoscope to measure the freezing point depression, determined the osmotic pressure of boar seminal plasma to be -0.55°C.

Although spermatozoal survival is greatest in isotonic diluents (Melrose 1962), spermatozoa can tolerate a considerable deviation from isosmotic levels (Emmens & Blackshaw 1956). Pronounced osmotic shock will, however, produce bending of the spermatozoal tails and induce circular swimming patterns, as well as high mortality (Foote 1969).

The effect of osmotic pressure on the motility of boar spermatozoa in vitro was reported by Salisbury (1962), Stevermer, First & Hockstra (1964), King and Macpherson (1966). Stevermer et al.(1964) concluded that motility was superior when slightly hypotonic diluents were used, but fertility was better in diluents isotonic with seminal plasma. King et al.(1966) reported that they could detect no difference in percentage motile spermatozoa, after freezing and thawing in diluents of different osmotic pressures.

Diluents as an energy source The metabolism of semen from different species was reviewed by Mann (1964) and boar semen by Aalbers et al. (1961), and Nevo et al.(1970).

Glucose is included as an essential ingredient for almost all diluents used for boar spermatozoa. The provision of an extracellular source of energy provides a means of sustaining metabolism in the anaerobic or semi-anaerobic conditions that occur during the laboratory storage of semen (Radford 1961).

The glycolytic breakdown of exogenous sugars in boar spermatozoa stored under anaerobic conditions, does not support any useful motility, in contrast to bull or human spermatozoa (Nevo et al.1970). This is possibly because boar spermatozoa cannot obtain sufficient energy for

motility from the anaerobic breakdown of fructose or glucose alone (Aalbers et al, 1961). The oxidative phase of sugar breakdown is probably the essential one, serving as a source of energy for ATP synthesis and supplying essential components for the metabolic machinery (Nevo et al, 1970).

Boar semen differs from that of other species in regards to motility and survival. When cooled to 15 - 20°C., the spermatozoa become immotile and regain motility on rewarming to body temperature after shaking in air (Polge 1956). The inter-relationship of temperature, aerobic and anaerobic storage conditions, and the consumption of fructose and glucose for sperm metabolism, is important in determining a suitable environment for the storage of boar spermatozoa in vitro.

Bacterial control The artificial insemination of pigs is promoted as a method of spreading genetic material without the risk of transmitting infectious desease. It is therefore important that there is no pathogenic bacteria in diluted semen.

The number of bacteria in boar semen at the time of ejaculation ranges from 5,000 to 2,200,000 per ml (Tanka, Niwa, Mizuho & Yoshida 1951). When added to diluents, these bacteria are placed in a good culture media, and may produce products toxic to the spermatozoa. They may also infect the female inseminated.

Bacterial contamination of semen can be minimised by first reducing the number of bacteria present in the ejaculate that is collected from the boar, then by adding antibiotics to the diluent to reduce the number further, during the period of storage.

The boars used on an artificial insemination centre are subject to regular health checks. They are screened for foot and mouth disease, swine vesicular disease, tuberculosis, leptospirosis (8 strains), Aujesky's disease and transmissable gastro enteritis.

Strict hygiene precautions are observed during the collection routine, and the pre-sperm fraction of the ejaculate that contains most of the bacteria is discarded.

The addition of antibiotics to the diluent not only prevents bacterial growth, but is also instrumental in maintaining a high level of fertility. Polge & Rowson (1956) obtained an improved farrowing rate and litter size when antibiotics were added to the diluent before dilution of the semen.

Melrose (1962) reviewed the addition of anti-bacterial substances to diluents used for cattle artificial insemination. These include penicillin, streptomycin, oxytetracycline, chlorotetracycline, chloromycetin, neomycetin and polymyxins. The antibiotics in common use for the addition to boar semen diluents are penicillin and streptomycin, these are used at the rate of 500 i.u. penicillin and 500 µg of streptomycin per ml of diluent.

Methods of semen storage.(Fresh v frozen)

At present there are two distinct methods being used for the storage of boar semen. The first method involves the use of 'fresh' or 'liquid' semen; the spermatozoa are diluted in a diluent and stored at a temperature above 4°C, then used within a few days from being collected. The second method involves the deep freezing of the spermatozoa and storing in liquid nitrogen at -196°C, where they may retain their fertilizing ability for several years.

In the United Kingdom, 'fresh semen' is used for routine artificial insemination. Well developed postal and railway services enable 'fresh' semen to be delivered to any part of the Country within 24 hours.

In the United States and Canada greater importance is attached to the use of frozen semen because of the great distances between farms and the less adequate railway and postal services. Climatic conditions also fluctuate in the United States and Canada. High Summer temperatures and low Winter temperatures make the constant storage temperature required for 'fresh' semen, difficult to maintain while in transit.

Within the United Kingdom, the primary advantage of using 'fresh' semen is that a high conception rate of up to 80 % with over ten pigs born per litter, can be achieved. Whereas with frozen semen the average conception rate is nearer 50 % with a litter size of six pigs born.

The semen from some boars 'freezes' better than that from other boars. At present it is difficult to identify the better fertility boars on the basis of semen examination alone. With 'fresh' semen the variation between the fertility of boars is considerably lower.

Diluting 'fresh' semen in an extender solution is a simple

procedure, requiring the minimum of laboratory equipment. Freezing procedures, at present, are more complicated and require considerable capital expenditure on equipment, particularly for the liquid nitrogen storage cylinders

A single dose of frozen semen requires two or three times the number of spermatozoa required for one dose of 'fresh' semen. With frozen semen, therefore, fewer doses are available from each ejaculate.

There are, however, several distinct advantages in the use of frozen semen related to long term storage. Long term storage enables semen supplies to be built up from valuable boars. The constant availability of semen from a boar facilitates the provision of a 'nominated' service, where a breeder can have semen on request from a particular boar.

Several countries insist on semen being held in quarantine before being imported, so that long term storage of semen is essential to the import and export trade. This method of storage also enables the importer, or any purchaser, to hold the semen until the females come into oestrus, thus avoiding the need to synchronise oestrus to coincide with the arrival of the semen.

THE DILUTION AND USE OF FRESH SEMEN

Introduction

The simplest method of utilising semen from boars for artificial insemination is to divide the ejaculate into 6 - 10 equal portions, immediately after collection from the boar, each portion being used for one insemination. The principal drawback to this technique is the relatively short life of the sperm cell, which, when stored in seminal plasma alone, will remain viable for only a few hours.

To prolong the storage period necessitates diluting the semen in a buffer solution (diluent), the primary function of the diluent being to buffer the spermatozoa against the products of metabolism and to provide an extracellular source of energy.

Semen diluents

A wide range of chemicals and organic compounds have been used in diluents for boar semen. These include egg yolk with citrate or phosphate, milk in its various forms, glucose, honey, saline solutions, glycine, saccharose and various combinations of salts (Rowson 1962).

The principal workers who have studied diluents for boar semen include Milovanov (1934); Selivanova (1934); Ito, Niwa, Kudo & Mizuho (1948); Noll (1950); Polge(1956); Du Mesnil du Buisson (1956); Aamdal & Hogset (1957); Du Mesnil du Buisson & Dauzier (1959); Radford (1961); Plisko (1965); Arhipovec (1966a); Balašov & Silaeva (1967); Poole, Foley & Plotka (1973).

In the early 1960's a pig artificial insemination service was established in the U.K. by the Pig Industry Development Authority. The diluent selected for use by this organization for processing 'fresh' semen, and subsequently used by other A.I. centres for the next ten

years, was the Illini Variable Temperature (IVT) diluent.

IVT diluent was originally developed for the storage of bull semen at room temperature (VanDemark & Sharma 1957) and incorporated egg yolk. This was later modified by excluding the egg yolk and used for the storing of boar semen at 15 - 20°C.

Constituents of IVT diluent

Chemical	Weight per litre of diluent
Tri-Sodium Citrate	20.0 gms
Sodium hydrogen carbonate	2.1 gms
Potassium chloride	0.4 gms
Glucose	3.0 gms
Sulphanilamide	3.0 gms
Penicillin	500,000 i.u.
Streptomycin	500 mg

The constituents are dissolved and made up to one litre with glass-distilled water.

Saturation of the diluent with CO_2 gas extends the storage life of the spermatozoa from 24 to 72 hours (Du Mesnil du Buisson & Dauzier 1959; Du Mesnil du Buisson & Jondet 1961). The mechanism of the CO_2 gas's effect on the spermatozoa is not clear, it appears to be of importance in initiating glycolysis, when added to IVT diluent, it assists in providing a suitable environment in which the spermatozoa can survive and metabolise (Lodge, Graves & Salisbury 1967; Shelby & Foley 1966; Sandford & King 1972).

The success of gassed IVT diluent is illustrated by its extensive use until the early 1970's when it was superseded by the EDTA diluent.

With the increase in demand for boar semen and the expansion of the artificial insemination service, came the impetus to develop a new diluent for the storage of liquid semen. This would have

a longer life than the 72 hours provided for by the IVT diluent. The new diluent came with the introduction of ethylenediaminetetra - acetic acid disodium salt (EDTA) as a diluent constituent (Plisko 1965; Arhipovec 1966a, 1966b; Sadovnikova 1966; Oĭvadis & Reseètnikova 1966; Senegačnik 1967, 1969).

Constituents of EDTA diluent

Chemical	Weight per litre of diluent
Glucose	60.0 gms
Tri Sodium citrate	3.7 gms
Ethylenediaminetetra-acetic acid disodium salt	3.7 gms
Sodium hydrogen carbonate	1.2 gms
Penicillin	500,000 i.u.
Streptomycin	500 mg

The constituents are dissolved and made up to one litre with glass-distilled water.

The main features of the EDTA diluent are that it is easy to prepare, the chemical constituents are readily available, and the spermatozoa when diluted in this diluent will retain their fertilizing capacity for up to five days and possibly longer when stored at 15 - 20°C.

As with most diluents, it is difficult to attribute the role of each individual constituent in the EDTA diluent that enables the diluent successfully to complete its function. Arhipovec (1966b) investigated the physical-chemical changes in the boar semen stored in EDTA diluent and reported that together with the sodium citrate, the EDTA blocks the action of the bivalent metalic ions (Ca^{++}, Mg^{++} etc) which have an activating effect on the enzyme systems of the spermatozoa.

A further characteristic of the EDTA diluent is the high level

of glucose in solution. Radford (1961) studied the metabolic rate of boar semen with added fructose. He reported that the addition of fructose to semen prolonged the survival time of the spermatozoa under anaerobic storage conditions. He also observed that the longer storage proceeded the more widely divergent became the curves for fructose usage and lactic acid production when the results were presented graphically. This was unexpected, since theoretically 1 mg of fructose uptake is equivalent to 1 mg of lactic acid production. It does however, support the work of Mann (1951) who found that ram spermatozoa were capable of oxidising lactic acid to lactate which can act as a substrate for mammalian sperms.

Further investigations are required to determine if the high levels of glucose in the EDTA diluent are essential, the effect of replacing some or all of the glucose with fructose or another sugar, the effect of varying the quantities of the other diluent constituents and the addition of other low electrolyte buffers. The ultimate objective being to extend the storage time of fresh semen for at least seven days without any loss in the capacity to fertilize, thereby enabling the continuous availability of semen from the boar, when collected from at weekly intervals.

The use of fresh liquid semen in the United States and Canada is very limited and greater emphasis is given to freezing and storing the spermatozoa in liquid nitrogen. However, a current trend is for 'on the farm' use of artificial insemination. In the larger herds, the semen from an individual boar is collected, extended in a diluent and used to inseminate several sows within the herd, thereby restricting the number of boars required in the herd and better utilization of the existing stud.

At the United States Department of Agriculture, Beltsville, Maryland, a diluent was formulated and recommended for use by the

farmers who wish to practice 'on the farm' artificial insemination. This is called the BL1 diluent (Pursel, Johnson & Schulman 1973b).

Constituents of BL1 diluent

Chemical	Weight per litre of diluent
Glucose	29.0 gms
Sodium citrate	10.0 gms
Sodium hydrogen carbonate	2.0 gms
Potassium chloride	3.0 gms

The constituents are dissolved in one litre of distilled water.

Bariteau, Bussiere & Courot (1976) compared the dilution and storage of spermatozoa in BL1 diluent and IVT diluent. They concluded that spermatozoa stored in BL1 diluent were more motile and had a higher conception rate when used over a period of five days.

In Canada they prefer to store the fresh liquid semen at 5°C. For this, they use an EDTA/egg yolk diluent. The semen is collected from the boars, diluted at room temperature, before being cooled in a refrigerator to 5°C. It is then transported in a polystyrene container packed with ice cubes.

Constituents of the diluent used by Ontario Swine A.I.Association

Chemical	Weight per litre of diluent
Glucose	50.0 gms
Sodium citrate	3.0 gms
Ethylenediaminetetra-acetic acid disodium salt	1.0 gms
Egg yolk	150 ml
Penicillin	500,000 i.u.
Streptomycin	500 mg

The constituents are dissolved in one litre of glass distilled water.

DEEP FREEZING OF BOAR SPERMATOZOA

Introduction

The long term preservation of boar spermatozoa is achieved by deep freezing and storing the spermatozoa in liquid nitrogen.

The discovery of glycerol as a cryoprotective agent reported by Polge, Smith & Parkes (1949) was a major advance in the field of semen preservation. Using glycerol as a 'protective agent' for freezing bull semen, one calf was produced when thawed semen was inseminated (Stewart 1951). Since then, rapid advances have been made in the techniques used for the preservation of bull semen leading to the establishment of a successful cattle insemination programme using deep frozen semen.

The successful freezing and thawing of boar semen has proved to be more difficult. In the early experiments by Roy (1955) and Polge (1956) when frozen-thawed boar spermatozoa was inseminated, based on the method used for bull semen was adopted, no pregnancies resulted.

After the first unsuccessful experiments, other investigations followed. A few reports of fertility following insemination of the frozen-thawed boar spermatozoa were published but even in the successful experiments the conception rates were low and the results could not always be repeated (Butler 1975).

In the early 1970's, consistently successful fertilization following insemination of frozen-thawed boar spermatozoa was reported by Crabo & Einarsson (1971); Graham, Rajamannan, Schmehl, Maki-Laurila & Bower (1971a, 1971b); Pursel & Johnson (1971a, 1971b, 1971c). Since then, other successful freezing procedures have been reported by Crabo, Brown & Graham (1972); Pursel & Johnson (1972b, 1975); Richter & Liedicke (1972); Salamon & Visser (1972, 1973); Vincente (1972); Wilmut & Polge (1972, 1977); Paquignon & Du Mesnil du Buisson (1973).

Of the early investigations, perhaps the most significant is the work undertaken by Graham at the University of Minnesota, St Paul, USA. Here, the basic techniques originated for some of the present procedures employed for freezing boar spermatozoa.

Graham et al.(1971a) reported having obtained a single litter of pigs through the use of frozen semen after over 300 sows had been inseminated without success, using semen frozen by various empirical techniques. However, the data accumulated led to significant advances in freezing procedures. In particular were the development of the TEST diluent, investigation on acceptable levels of glycerol, a study of the effect of 'holding time' on the freezability and enzyme release of boar spermatozoa and the inclusion in the diluent of a 'surfactant agent'.

TEST diluent

Graham et al.(1971a) formulated a diluent by titrating TES (N Tris (Hydroxy-methyl) methyl-2-Aminoethane sulphonic acid) with Tris (hydroxymethyl amino methane) to a pH of 7.0. Four percent by volume of isotonic fructose solution was added and twenty percent by volume of fresh egg yolk. (Appendix 1) The preparation was centrifuged at 12,000 g for 10 minutes and the supernatant used as the diluent under the name of TEST or TESNAK (Crabo, Brown & Graham 1972).

Glycerol concentration

The usefulness of glycerol as a cryoprotective agent has been established for bull semen but reports of the effect of glycerol on non-frozen boar semen have pointed repeatedly to a depressing effect on fertility (Polge 1956, King & Macpherson 1966, Neville, Macpherson & King 1970, Wilmut & Polge 1974).

Graham et al.(1971a) studied the effect on non-frozen boar semen of different levels of glycerol. They reported no difference between the control (no glycerol) and 2 % glycerol in the diluent.

Higher levels of glycerol drastically reduced fertility.

A further study by Graham et al.(1971b) indicated that when using the TEST diluent, the addition of glycerol to frozen semen was positively detrimental. Of 24 sows served with frozen semen containing no glycerol, 11 (45.8 %) were pregnant, with frozen semen containing 1.0 % glycerol only 4 of 26 (15.4 %) were pregnant, while none of 26 sows inseminated with frozen semen containing 7.0 % glycerol became pregnant.

The effects of semen dilution with glycerol on the release of Glutamic-oxaloacetic transaminase (GOT) from the sperm cell was studied by Bower, Crabo, Pace & Graham (1973). The purpose of the study was to assess cell damage by assessing enzyme leakage from the cell, GOT being selected as the most suitable enzyme. From the results of their investigations Bower et al.(1973) concluded that glycerol damaged the spermatozoa regardless of when or how it was added, and that the damage was proportional to the concentration of glycerol added.

Sandford, King & Macpherson (1971) studied the influence of glycerol concentration and freezing to - 79°C on the oxygen uptake and motility of both bull and boar spermatozoa. They reported that the presence of glycerol appeared to have a more detrimental effect on the respiration of boar spermatozoa than on that of bull spermatozoa. Boar spermatozoa used up to 43 % less oxygen in the presence of 7.5 % glycerol. Bull spermatozoa, however, took up only 21 % less oxygen in the presence of the same level of glycerol during the 1st hour of incubation after thawing, suggesting that the boar spermatozoa surviving the freezing process metabolise at a much lower rate than normal. They reported also that the active progressive motility of most of the surviving boar spermatozoa ceased within 1 to 2 hours of post-thaw incubation. (Progressive motility - forward movement of

spermatozoa as opposed to circular or backward movement).

Sandford et al.(1971) used a skim milk, egg yolk, glucose diluent, the results of their experiments could be attributed to the inability of the diluent adequately to buffer the spermatozoa against cold shock, as much as to the detrimental effect of glycerol. Other diluents would certainly enable a considerably longer period of progressive post-thaw motility of the spermatozoa.

The detrimental effects of glycerol at low concentrations, in boar semen diluents were also investigated by Butler (1975) and Johnson & Pursel (1976).

Butler (1975) reported that the condition of the cooled, but unfrozen boar spermatozoa were not affected by the level of glycerol present in the diluent. Also the percentage of motile spermatozoa, after freezing and thawing, increased when the glycerol concentration was increased from 0 to 4 %.

Johnson & Pursel (1976) reported that the presence of 1 % glycerol in the BF5 diluent, caused an increase in post-freeze-thawed motile spermatozoa, motility was 29 % in samples containing 1 % glycerol compared to 20 % in samples containing no glycerol. BF5 diluent was developed from the TEST diluent by Johnson and Pursel. At present, it is the most popular freezing diluent, being used extensively in North America and to a lesser extent in Europe. As part of the BF5 freezing procedure, glycerol is added to the diluted semen after cooling, and immediately before freezing, to give a final concentration of 1 % in the diluted semen mixture. (Appendix 4)

Johnson & Pursel (1976) also measured the loss of acrosin from the sperm cell, using this an indicator of damage to the cell when subjected to the freezing and thawing procedure. They concluded that release of acrosin from the cell was not affected by the inclusion of 1 % glycerol as a component of BF5 diluent.

The effect of 'holding time'

Graham et al.(1971a, 1971b) reported on the beneficial effect of 'holding time'. This is the period after collection of semen from the boar and before dilution. The imposition of a period of 'holding time', which may extend from zero to 6 hours, results in an increase in post freeze-thaw motility and a reduction of damage to the cell, as measured by GOT loss with time from the sperm cells.

One explanation of this phenomenon is that after ejaculation, the membranes of the spermatozoa are strengthened as a result of the coating of the spermatozoa by the seminal plasma proteins. This beneficial effect may be lost when the egg yolk protein is added prematurely to the semen (Graham et al.1971a) It is, however, far from clear what effect the seminal plasma proteins do have on the spermatozoa in vitro . The effects on spermatozoa of seminal plasma proteins have been investigated by Moore (1975); Pavelko & Crabo (1976); Moore & Hibbett (1976, 1977); Moore, Hall & Hibbitt (1976).

The results of the investigations of Moore et al.(1977) indicate that spermatozoa from boars without seminal vesicles (i.e. vesiculectomised boars) gave conception rates not significantly different from those obtained with spermatozoa from intact boars. The viability of the spermatozoa before and after freezing, as measured by GOT enzyme release, motility and percentage eosinophilic spermatozoa, was apparently the same for all animals, no difference being observed between the vesiculectomised and the intact animals.

Pursel, Johnson & Schulman (1973a) assessing the damage to the spermatozoa using acrosome morphology and sperm motility, reported that spermatozoa when washed before incubation (holding time) were more resistant to cold shock after five hours 'holding time' than after one hour 'holding time'. This suggests, that the acquisition of resistance

to cold shock during the 'holding time' period was an inherent property of the spermatozoa. However, they concluded that seminal plasma did provide boar spermatozoa with some additional protection against cold shock.

Pavelko & Crabo (1976) used radioactively (^{125}I) labelled boar seminal plasma proteins to investigate the association of boar seminal plasma proteins with boar spermatozoa. They demonstrated that during the holding time, the radioactivity was immediately detectable on the spermatozoa and continued to increase for two hours. A comparison between the semen from a fertile and that from an infertile boar showed a sharp contrast in the uptake and maintenance of radioactivity on the sperm cell. The spermatozoa from the infertile boar accumulated only low amounts of radioactivity, most of which was lost during the thawing procedure. The authors suggested that the inability of spermatozoa to accumulate seminal proteins may be a reason for impaired fertility

The addition of a surfactant to egg yolk diluent

Graham et al.(1971a, 1971b) screened seventy eight surfactants for their effectiveness on the maintenance of the cell membrane and on the motility of boar spermatozoa. They reported that the addition of Orvus es paste (OEP), a synthetic detergent and wetting agent, to the semen diluent, decreased the freeze-thaw damage to the spermatozoa. Subsequently OEP has been incorporated into other boar semen diluents (Pursel & Johnson 1975).

The beneficial effect of OEP on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar spermatozoa was reported by Pursel, Schulman & Johnson (1977). They found that the acrosomes of boar spermatozoa, diluted in egg yolk diluent containing 0.5 % OEP, were better able to withstand the freeze-thaw process than spermatozoa

in diluent without OEP.

The mechanism of the surfactant's protection is unknown, but the observation that the incubation of boar spermatozoa with as little as 0.1 % OEP in a diluent devoid of egg yolk, had a marked deleterious effect on acrosome morphology and spermatozoan motility, would suggest that the beneficial action of the OEP is not on the sperm cell but on the egg yolk.

Freezing procedures

Four current methods of freezing boar semen i.e. TEST, Vincente, Hülsenburg and BF5 techniques are given in appendices 1,2,3,& 4. A comparison of three of these methods, TEST, Hülsenburg and BF5 was made by Butler (1975). She reported that the results obtained with semen frozen by each of these techniques was encouraging, in that some fertility was obtained with each, following intracervical insemination. The major drawback was the variation in fertilization results when semen from different boars were used for inseminations, indicating that semen from certain boars have poor freezing qualities. A further drawback is the low number of inseminations available from each ejaculate when frozen. Some ejaculates produce only one or two inseminations; this, when combined with low conception rates, limits the economic feasibility of using frozen semen.

Continuing research into the freezing of boar semen will enable constant improvement of the current methods, so that in the not too distant future the results of using frozen semen should become comparable to those obtained using fresh semen.

EXPERIMENT II

Acrosome Morphology Alteration During the BF5 Freezing Procedure

Introduction

The effects of deep freezing upon the morphology of the spermatozoa, and in particular on the acrosome cap, have been well documented with boar spermatozoa (Pursel, Johnson & Rampacek 1972; Pursel, Johnson & Schulman 1972c, 1973a). However, during the several stages of cooling and freezing boar spermatozoa, certain technical procedures have to be followed i.e. holding time, centrifugation dilution, cooling to 5°C, addition of diluent with glycerol at 5° C and pelleting on a dry ice block (Appendix 4).

The effects of these procedures on the acrosome up to the actual immersion in liquid nitrogen have not been widely reported. This experiment was therefore designed to determine the changes to the acrosome cap of the spermatozoa associated with these preliminary technical procedures. An additional experiment was conducted to determine the changes in acrosome morphology associated with pellet size and with the time the pellets actually remain on the dry ice block.

Experiment 1

Objective To determine the damage to the spermatozoal acrosomes during the stages involved when cooling to 5°C.

Materials and method Semen from the sperm rich fraction of six boars was collected and cooled following the method of Pursel & Johnson (1975).

Samples of the spermatozoa were taken at several stages during the cooling procedure as follows:

Sample 1 - after ejaculation.

Sample 2 - after 2 hours 'holding time'.

Sample 3 - after centrifugation and reconstitution in BF5 diluent.

Acrosome Morphology using a Phase-Contrast Microscope

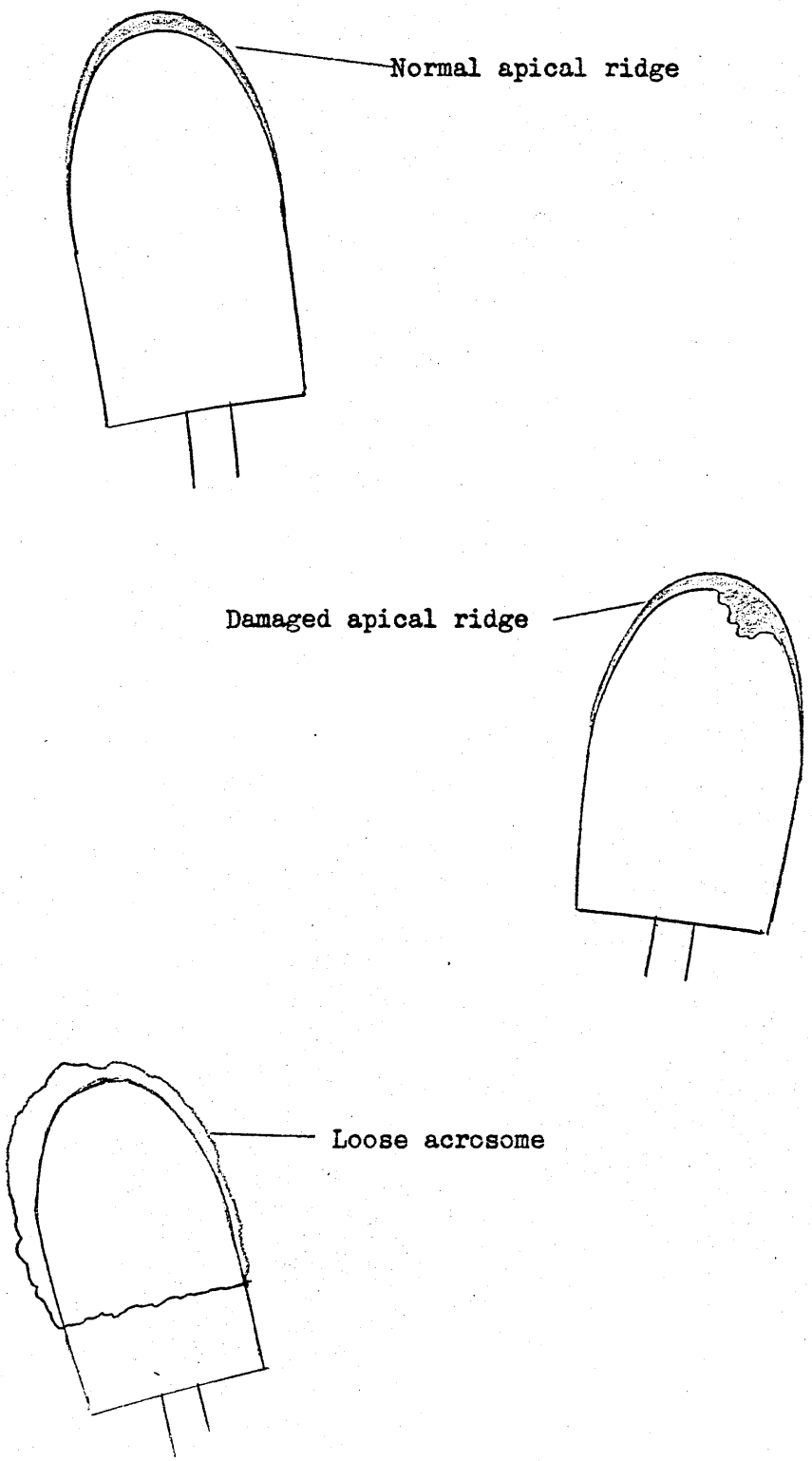


Fig 11

Sample 4 - after cooling to 5°C.

Sample 5 - after the addition of diluent with glycerol of 5°C.

The samples were fixed using glutaraldehyde (Appendix 5). Samples 4 and 5 were rewarmed to 20°C before fixation. A fraction of sample 5 was fixed at 5°C; this was then designated sample 6.

A drop of the fixed semen from each sample was placed on a glass slide under a cover slip and examined for acrosomal damage, using phase-contrast microscopy at x 1,000 magnification. Two slides were examined for each sample, with 100 acrosomes evaluated on each slide.

The acrosomes were categorized as having a normal apical ridge (NAR) if the acrosomal cap smoothly adhered to the nucleus and possessed a distinct apical ridge that formed a smooth crescent at the apical border of the head, as described by Pursel, Johnson & Rampacek (1972) and illustrated by fig.11.

Results

The % of spermatozoa displaying Normal Apical Ridges (NAR) in the semen of six boars. The samples taken at different stages during the cooling procedure

	% NAR of semen from 6 boars						Mean
Sample 1	95	94	89	97	97	100	95
" 2	96	93	95	98	98	98	96
" 3	83	85	94	96	96	99	92
" 4	86	90	88	94	92	85	89
" 5	68	83	89	92	94	88	85
" 6	78	89	88	93	86	94	88

Conclusion

There is no significant difference in the number of spermatozoa displaying normal apical ridges in the samples taken after ejaculation from the boar and the samples taken at stages during the cooling of the spermatozoa to 5°C.

Experiment 2

Objective To determine the damage to spermatozoal acrosomes during the stages of freezing on dry ice and in liquid nitrogen.

Materials and method Samples of semen from the sperm rich fraction of the ejaculates from five boars were used. An additional sample of semen was obtained by mixing part of the ejaculates from three of the boars. The six samples were diluted, cooled and frozen following the BF5 procedure (Appendix 4).

Further samples were taken from each of the diluted semen samples for morphological examination as follows:-

Sample 1 - after cooling to 5°C and the addition of diluent with glycerol.

Sample 2 - after freezing on a CO_2 ice block, then thawed in a thawing solution (Appendix 5).

Sample 3 - after freezing on a CO_2 ice block, immersion in liquid nitrogen and thawed in a thawing solution (Appendix 5).

The spermatozoa from samples 1, 2 and 3 were fixed using glutaraldehyde and examined by phase-contrast microscopy for NAR as in experiment 1.

Results

The % of spermatozoa displaying normal apical ridges. The samples taken at different stages during the freezing procedure

	% NAR of spermatozoa						Mean
Sample 1	93	93	90	90	96	95	93
Sample 2	38	26	16	24	27	37	28
Sample 3	27	4	14	21	24	32	20

Conclusion Damage to the spermatozoal acrosome appears to occur during the stage of cooling on the CO_2 ice block from 5°C to - 40°C.

Discussion Further experiments are called for to investigate the factors that could affect the damage to the acrosome when freezing on the CO_2 ice block. A possible influence could be the size of the pellet that is frozen; the smaller the pellet the faster will be the rate of cooling. The smaller pellet will also have a relatively greater surface area per volume of semen.

A further factor that could influence the extent of damage to the spermatozoa during the initial freezing period to - 40°C is the length of time the pellets remain on the dry ice block before being plunged into liquid nitrogen.

These possible causes of acrosome damage are investigated in experiments 3 and 4.

Experiment 3

Objective To determine the effect of pellet size on damage to the spermatozoal acrosome during the period of freezing on a CO_2 ice block.

Materials and method Semen was collected from two boars, diluted and cooled to 5°C following the BF5 procedure (Appendix 4). Pellets were made of five different volumes. This was achieved by selecting a pasteur pipette, from which one drop was 0.05 ml. By regulating the number of drops it was possible to make a series of pellets on the ice block, ranging in size, from 0.05 to 0.25 ml.

The pellets were allowed to remain on the ice block until frozen before being thawed in thawing solution at 50°C. Samples of the spermatozoa from each of the pellet sizes were fixed with glutaraldehyde and examined by phase-contrast microscopy as in the previous experiments.

Results

The % of spermatozoa displaying normal apical ridges from diluted semen, frozen on a CO_2 ice block in pellet sizes ranging from 0.05 to 0.25 ml.

	% Normal apical ridges		
Pellet size	1st Boar	2nd Boar	Mean
0.05 ml	18	18	18
0.10 ml	28	37	32
0.15 ml	33	39	36
0.20 ml	23	27	25
0.25 ml	15	22	18

Conclusion The optimum pellet size for freezing diluted semen by the BF5 technique is 0.15 ml.

Experiment 4

Objective To determine the damage to spermatozoal acrosomes by varying

the period of cooling on the CO_2 ice block from 1 to 10 minutes.

Materials and method Semen was collected from two boars, diluted and cooled to 5°C following the BF5 procedure (Appendix 4). The diluted semen was placed on a CO_2 ice block to form 0.2 ml pellets. Samples were recovered at intervals after placing on the ice at 1,2,4,6, and 10 minutes. These pellets were thawed in thawing solution at 50°C, fixed in glutaraldehyde, and the spermatozoa were examined by phase-contrast microscopy for normal apical ridges as in experiment 1.

Results

The % of spermatozoa displaying normal apical ridges when cooled on a CO_2 ice block for periods of 1 - 10 minutes.

	% Normal apical ridges		
Time on ice block	1st Boar	2nd Boar	Mean
1 min	43	45	44 *
2 mins	30	27	28
4 mins	30	30	30
6 mins	25	21	23
10 mins	33	31	32

* semen only partially frozen

Conclusion After 1 minute the semen pellet is only partially frozen and damage to the spermatozoal acrosomes is less pronounced. Apart from this, the damage to the acrosomes does not appear to be related to the length of time, the pellet is frozen on the CO_2 ice block.

Discussion

The purpose of this series of experiments was to elucidate the effect of the various procedures involved in freezing boar semen

by the BF5 technique on acrosome morphology.

One of the major difficulties with freezing boar semen is in establishing a method of evaluating the viability of the spermatozoa after freezing and thawing. Certainly, the visual assessment of the motility of post-thawed semen is not a reliable indicator of fertility, and not much reliance can be placed on acrosomal abnormalities. The potency of semen ultimately depends on its ability to survive within the female tract, this being the crucial factor (Pursel - personal communication).

When evaluating the damage to spermatozoa by cold shock after freezing, the spermatozoa that can survive with the membranes intact, must have been afforded a greater protection than the spermatozoa with disrupted membranes. It is for this reason that acrosome counts of spermatozoa with normal apical ridges are used as an indicator of damage to the spermatozoa. In experimental work, the percentage NAR has some relevance within one ejaculate but not between ejaculates from different boars due to inter-boar variation (Pursel - personal communication).

No significant difference was observed in the number of spermatozoa displaying NAR in the samples taken after ejaculation and at the stages of cooling. This was encouraging as during the cooling period the spermatozoa are subjected to two dilutions, centrifugation and 'holding time'. Pursel et al.(1973a) reported that the percentage NAR in frozen-thawed semen was higher after a period of 5 hours 'holding time' than after a shorter period of 'holding time'. The seminal plasma apparently interacted with the spermatozoa during the 'holding time' to produce increased resistance to cold shock. Also, the dilution of semen during 'holding time' interferred with the resistance to cold shock. There appears to be considerable scope for further investigation on both the length of 'holding time' and the effect of

dilution rates. Centrifugation does not affect the percentage of NAR, which is in agreement with Pursel et al.(1973a).

Experiment 2 illustrated that the damage to the acrosomes appears to occur during the stage of cooling, on the CO_2 ice block, when the temperature of the diluted semen is lowered through freezing point to - 40°C. Although this experiment indicates when the spermatozoa are damaged, the cause of the damage is not apparent.

Experiment 3 investigated the effect of pellet size on acrosomal damage. Although the pellet size appeared to make some difference to the NAR, the major factor remains, that during the freezing process the spermatozoa are susceptible to damage. During the treatment prior to freezing, the spermatozoa had not developed the resistance to cold shock.

Further investigations are required to study why boar spermatozoa differ markedly in their response to cold shock in comparison to bull spermatozoa; why glycerol, egg yolk, milk, casein etc. will function as cryoprotective agents for bull semen but not to the same extent for boar semen; why boar spermatozoa when, after freezing and thawing, will display excellent motility with relatively high levels of normal acrosomes, yet, when used for insemination, result in low conception rates.

CONCLUSION

The production of spermatozoa by the boar is a result of the sequence of events that occur within the seminiferous epithelium of the testes. These events include the production of the spermatogonia from the stem cells, through the various stages of spermatogenesis, to the release of the spermatozoa within the lumen of the seminiferous tubule. The spermatozoa attain maturity in the epididymis, the caudal region of which also serves as a reservoir for the spermatozoa.

The developing germ cells in the seminiferous epithelium appear sensitive to a change in temperature caused by exposure of the animal to high ambient temperatures. The damage to the cells becomes apparent in the semen ejaculate some time afterwards and demonstrates the inability of the animal to reduce testicular temperature. This emphasizes the importance of keeping boars, used for artificial insemination, in environmentally controlled buildings. The results from Experiment I indicate there is no apparent difference in the conception rate, litter size and morphology of semen taken from boars, kept in purpose built housing, over the winter and summer months.

The collection of semen from the boar is a simple procedure; fortunately most boars have a high libido and can be trained to mount a dummy sow and ejaculate after fixation of the penis using the gloved hand technique. The frequency of semen collections is important so that the maximum number of spermatozoa can be harvested. It is interesting to note that Swierstra (1971) reported a close relationship between daily sperm production and daily sperm output when boars were collected from at 72 hour intervals; the sperm output averaged 99 % of sperm production. Further investigation is required to determine differences that may occur in sperm production rates according to breed, age and strain of boar.

As most of the bacteria in the semen ejaculate can be discarded

at the time of collection, further investigation of the collection technique would be useful to determine the effects of discarding fractions of the ejaculate, washing and shaving the prepuce etc. to reduce to a minimum the bacterial contamination of the collected semen.

Considerable work has been undertaken on the assessment of semen 'quality' by laboratory methods. It is difficult to correlate any particular examination technique with conception rate. This applies particularly to deep frozen semen. One of the major drawbacks to the present methods of freezing semen is the individual boar differences; semen from some boars will withstand the freezing procedure better than semen from other boars. The present method to distinguish the boars that will produce semen that will freeze successfully, is to inseminate frozen semen from each boar in the stud and observe the conception rates. This is expensive, time consuming and to be effective must involve inseminating large numbers of pigs to obtain sufficient data. There is a rapid turnover of boars on an artificial insemination centre; also, the composition of the semen of a boar changes as the animal ages (Johnson & Pursel 1971). So the value of this method is limited and further investigations are required to examine methods of assessing deep frozen semen to determine its potential fertility. One possible method is to measure the freezing-point depression of semen from individual boars (Polge-personal communication) to determine if there is a correlation between the freezing-point depression and the ability of the semen to withstand the freezing and thawing procedures. Another method would be to use more sophisticated laboratory apparatus such as chemical analysers, spectrometers and sensitive pH meters, to measure finite differences in semen composition from individual boars to determine if there is a correlation between these measurements and conception rates.

The freezing and storage of boar spermatozoa in liquid nitrogen, is at present of only limited economic value in the United Kingdom, its main importance being in the export of semen. This situation could change if the present disadvantages could be overcome.

The use of fresh semen diluted in EDTA diluent enables the storage time of viable spermatozoa to be extended for up to five days after ejaculation when stored at a temperature between 15 - 20°C. To prolong the life of the spermatozoa for seven days would be advantageous; a week's supply of semen could be dispatched to the farmer, reducing the administration and distribution costs. To achieve a seven day diluent would require further investigation of the present system of storage, including the effect of varying the amounts of the existing constituents of the diluent, the addition of other low electrolyte buffers, and the possible effect of lowering the storage temperature to suppress metabolism of the spermatozoa.

Research on the production and preservation of boar semen has developmed with the expansion of the use of boar semen for artificial insemination. The continued expansion of artificial insemination is inevitable, as the emphasis is changed from being used to introduce new genetic material into a herd, to its use for the production of slaughter generation pigs. This expansion of artificial insemination services, accompanied by improved techniques and methods for the preservation of boar semen should eventually transform the pig industry in much the same way the cattle industry has been transformed.

APPENDIX 1

Method of freezing boar semen - TEST Method

References: Graham et al.(1971a, 1971b); Butler (1975).

Preparation of diluent

The diluent is prepared by titrating a solution of Tes (N Tris(Hydroxy-methyl)methyl-2-aminoethane sulphonic acid) (7.4 gms /100 ml) with Tris (hydroxymethyl amino methane) (3.9 gms/100 ml) to pH of 7.0. To 70.5 ml of the tes/tris mixture is added 4 ml of fructose solution (5.6 gms/100 ml); 5 ml sodium citrate solution (5.6 gms/100 ml); 0.5 ml orvus es paste and 20 ml of egg yolk.

Cooling and freezing

The sperm rich fraction of boar semen is collected and allowed to cool to 22°C over a period of one hour, then maintained at 22°C for an additional hour. The semen is diluted (1 : 5) with TEST diluent and further cooled to 5°C over a four hour period. The cooled semen mixture is frozen by the pellet method (Nagase, Graham and Niwa 1964) and stored in polythene bottles in liquid nitrogen.

Thawing

The pellets are thawed by scattering on a Teflon-coated pan, warmed to 15°C. When the pellets have almost completely thawed they are poured into a container, held at room temperature and used for the insemination within 15 minutes.

APPENDIX 2

Method of freezing boar semen - Vincente method

Reference; Vincente (1972)

Constituents of diluents

Diluent 1.	Sodium citrate	20.77	gms
	Glucose	3.00	gms
	Sodium bicarbonate	2.00	gms
	Potassium chloride	0.01	gms
	Distilled water	1,000	ml
Diluent 2.	Powdered milk	10.0	gms
	Egg yolk	30	ml
	Streptomycin sulphate	50	mg
	Penicillin	50,000	i.u..
	Polymixin sulphate	5	mg
	Distilled water	100	ml

Note: The powdered milk should contain less than 0.3 % fat and a maximum of 0.6 % total solids.

Diluent 3	Glucose	10.0	gms
	Sodium citrate	6.0	gms
	Sodium bicarbonate	1.2	gms
	Potassium chloride	1.5	gms
	K_2HPO_4	5.0	gms
	Citric acid	1.5	gms
	Distilled water	1,000	ml

Cooling and freezing

The sperm rich fraction is collected from the boar and mixed with an equal volume of diluent 1. It is allowed to cool and then stored for 2 hours at 14 - 20°C before centrifugation at 2,500 r.p.m. for 20 - 25 minutes.

The supernatant is discarded and the sediment diluted at the rate of 1:3 or 1:5 with diluent 2. The temperature of the diluted semen is reduced by 1°C per minute until it reaches 2 - 3°C and held at this temperature for 4 hours.

Glycerine is added to the diluted semen to a final concentration of 6 %, before being placed in 0.5 ml straws and allowed to equilibrate for 16 - 20 hours at 2 - 3°C.

After equilibration the straws are exposed to liquid nitrogen vapour for 25 minutes before being totally immersed in the liquid nitrogen.

Thawing

5 - 8 Straws are used for each insemination. These are thawed by removing from the liquid nitrogen and placed in 70 - 100 ml of diluent 3 at 37°C.

APPENDIX 3

Method of freezing boar semen - HULSENBERG method

References: Richter & Liedicke (1972); Butler (1975)

Constituents of diluent

Glucose	6.0 gms
Sodium citrate	0.6 gms
EDTA disodium salt	0.125 gms
Sulphanilamide	0.1 gms
Sodium bicarbonate	0.125 gms
Potassium chloride	0.075 gms
Penicillin	100,000 i.u.
Streptomycin	8.5 mg

The constituents are dissolved and made up to 100 ml with distilled water. Before use, the diluent is saturated with carbon dioxide and the pH adjusted to 6.8 using a 4 % solution of sodium hydroxide.

Cooling and freezing

The sperm rich fraction of the ejaculate is collected from the boar and diluted either 1:2 or 1:3 with the above diluent and cooled over a 6 hour period to 15°C.

The diluted semen is centrifuged at 1,000 to 2,000 r.p.m. for 8 minutes and the supernatant discarded. The sediment is resuspended in a similar diluent to the one already described, without the carbon dioxide, but including 10 gms of dried skim milk powder, 18 ml of egg yolk and 5 ml of glycerol per 100 ml. The resuspended spermatozoa are cooled to 5°C over a period of 1 to 2 hours, then pelleted on dry ice before being transferred to liquid nitrogen.

Thawing

To thaw, the pellets are tipped into a vessel containing 50ml of the thawing diluent (same as freezing diluent excluding CO_2) held in a water bath at 50°C.

APPENDIX 4

Method of freezing boar semen - BF5 method

Reference: - Pursel & Johnson (1975)

Preparation of diluent (BF5)

Tes	1.2	gms
Tris	0.2	gms
Glucose	3.2	gms
Egg yolk	20.0	ml
Orvus paste	0.5	ml

The tes, tris and glucose are dissolved in warm distilled water and the volume made up to 79.5 ml. 0.5 ml of orvus paste is added, followed by 20 ml of egg yolk. The diluent is centrifuged at 4,000 r.p.m. for 20 minutes and the sediment discarded. The supernatant is divided into two equal volumes, one part is used at room temperature for the first dilution. After 2 % glycerol has been added to the remainder it is cooled to 5°C in preparation for the 2nd dilution.

Preparation of thawing solution (BTS)

Glucose	37.0	gms
Sodium citrate	6.0	gms
Sod. bicarbonate	1.25	gms
EDTA	1.25	gms
Potassium chloride	0.75	gms

These constituents are dissolved and made up to 1,000 ml with distilled water.

Cooling and freezing

The sperm rich fraction of the semen is collected from the boar and allowed to cool to 20°C in an unstoppered flask for two hours. During this 'holding' or 'equilibration' period, 2,000 mg of streptomycin sulphate are added per ml of semen.

After 2 hours the semen is centrifuged at 1500 r.p.m. for 10 minutes, the supernatant is discarded. The semen pellet in the centrifuge tube is resuspended with BF5 diluent without glycerol, to give a concentration of 5×10^9 sperm per 5 ml. This semen mixture is cooled to $5^{\circ}C$ over 2 hours, after which the 2nd BF5 diluent with 2 % glycerol is added at a 1:1 ratio. The final dilution giving a concentration of 5×10^9 sperm per 10 ml. Immediately after the 2nd dilution the cooled semen is frozen into pellets on a dry ice block, the pellets are then transferred to liquid nitrogen.

Thawing

The frozen semen pellets are thawed by tipping them directly into BTS thawing solution held at $50^{\circ}C$ in a water bath. Each 10 ml of pellets is thawed in 50 ml of BTS thawing solution. This volume is sufficient for one insemination.

APPENDIX 5

Procedure for thawing and fixing deep frozen semen for acrosomal examination

Preparation of thawing solution

Glucose	3.7	gms	Dissolved in distilled water and made up to 100 ml
Sodium citrate	0.6	gms	
Sod. bicarbonate	0.125	gms	
EDTA	0.125	gms	
Pot. chloride	0.075	gms	

Preparation of fixative solution

Lactose	4.0	gms	Dissolve in distilled water and made up to 100 ml
Fructose	0.5	gms	
Tris	2.0	gms	
Glutaraldehyde (50 % sol.)	4.0	ml	

Thawing Three pellets are removed from the liquid nitrogen and thawed in a test tube containing 1.0 ml of thawing solution at 50°C. When the pellets have thawed the test tube is suspended in a water bath at 30°C.

Fixation Using a pasteur pipette, six drops of thawed semen are pipetted into 0.5 ml of fixative solution. The fixative solution should be maintained at the same temperature as the semen.

Examination A drop of the fixed semen is placed on a glass microscope slide under a cover slip. The spermatozoa are examined for acrosomal damage by phase-contrast microscopy at x 1,000 magnification.

APPENDIX 6

The use of nigrosin/eosin stain for the morphological examination of spermatozoa

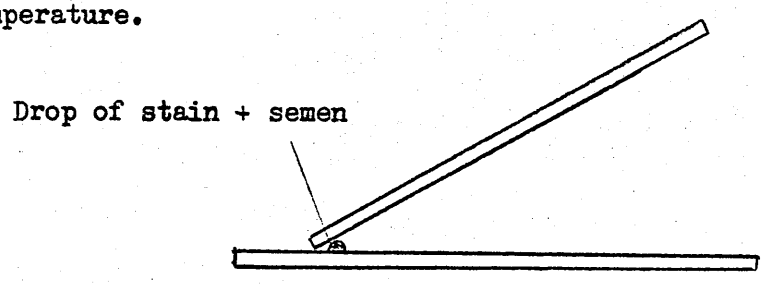
Reference: Campbell, Hancock & Rothschild (1953)

Preparation of stain

Sodium citrate	2.90	gms
Nigrosin	10.00	gms
Eosin	1.77	gms

Sodium citrate is dissolved in 100 ml of warm tap water. The nigrosin and eosin are dissolved in the sodium citrate solution by heating for 30 minutes in a flask, suspended in boiling water. The stain is then filtered to remove any undissolved particles.

Preparation of stained semen smear It is important that both semen and stain are the same temperature, to ensure this, both should be suspended in a water bath at 28 - 30°C. Using a pasteur pipette, six drops of stain are placed in a test tube and 2 - 6 drops of semen are added according to the density. The stain/semen solution is shaken to mix, and left for 5 minutes before a drop is placed on a clean microscope slide. A second slide is used to draw the stained semen across the slide to give an even smear that should dry quickly at room temperature.



Examination The smears are examined under the light microscope at x 800 magnification. To ensure a representative sample of the sperm are counted, a succession of fields are read diagonally across the smear. Two slides are prepared for each sample of semen and 100 spermatozoa are examined on each slide.

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